University of Alberta

Development and Applications of Bispecific Antibodies

by

Ying Cao ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial
fulfillment of the requirement for the degree of Doctor of Philosophy

in

Pharmaceutical Sciences

Faculty of Pharmacy and Pharmaceutical Sciences

Edmonton, Alberta

Spring 1999

National Library
of Canada

Acquisitions and
Bibliographic Services

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale
du Canada

Acquisitions et
services bibliographiques

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-39510-3

Canada

University of Alberta

Library Release Form

Name of Author: Ying Cao

Title of Thesis: Development and Applications of Bispecific Antibodies

Degree: Doctor of Philosophy

Year This Degree Granted: 1999

Permission is hereby granted to the University of Alberta Library to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly, or scientific research purpose only.

The author reserves all other publication and other rights in association with the copyright in the thesis, and except as hereinbefore provided, neither the thesis nor any substantial portion thereof may be printed or otherwise reproduced in any material form whatever without the author's prior written permission.

Cao

425 N. Charter St.
School of Pharmacy
University of Wisconsin-Madison
Madison, WI 53706

Date Jan. 6, 1999

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Development and Applications of Bispecific Antibodies submitted by Ying Cao in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Pharmaceutical Sciences.

Dr. M.R.Suresh (Supervisor)

Antoine Noujaim
Dr. A.A. Noujaim (Co-Supervisor)

Dr. S. McQuarrie (Committee Chair)

Dr. J. Samuel (Committee Member)

Dr. L.I. Wiebe (Committee Member)

Dr. A.J.B. McEwan (Committee Member)

for Dr. A.C.G. Cuello (External Examiner)

Date Jan 6th 1999

Dedicated

to

My dear parents, Guoyin Cao and Shufen Zhang. Without their love and support, this thesis will never be finished.

Abstract

Bispecific antibodies are unique macromolecular heterobifunctional cross-linkers with two different binding specificities. As unique bioconjugates, they have immense potential in biological and immunological fields. The long-term goal of this research is to develop such useful bioconjugates incorporating a biotin binding arm, eliminating the need for chemical conjugations in a variety of applications.

First, a hybridoma secreting MAb against biotin has been generated by conventional hybridoma technology. Then, a novel non-selective micro-electrofusion method was developed to generate two quadromas secreting bsMAb with anti-biotin paratope. The bsMAb (anti-biotin x anti-HRPO) has comparable avidity to commercial streptavidin-HRPO when tested against biotinylated macromolecules. BsMAb can bind the enzyme directly and thus eliminate the need for chemical conjugation. This bsMAb has been used as a promising immunoprobe for detecting many macromolecules bearing biotin markers, such as protein, phage, liposome and DNA in different bioassay systems. Finally, a tumor-specific drug delivery system has been developed combining pretargeted bsMAb (anti-biotin x anti-squamous carcinoma) and biotin pegylated liposome. This new strategy has superior properties for potential tumor therapeutics: (1) Pegylated liposomes could deliver a large amount of radioisotopes or anti-cancer drugs to the target. (2) Pretargeted bsMAb could minimize the limitations of immunoliposome applications *in vivo*. (3) Modification of liposomal surface by biotin is a relatively easy process and would not affect liposome

characteristics. (4) Multiple biotin moieties on the surface of pegylated liposome enable it to have much higher local biotin concentration compared to free biotin hapten when binding to anti-biotin binding arm of bsMAb. (5) Cross-linking multi-epitopes on the surface of a tumor cell could initiate endocytosis and intracellular delivery of cytotoxic drugs. These studies will open up new strategies in solid tumor therapeutics.

Acknowledgments

I would like to thank my supervisors Dr. M.R. Suresh and Dr. A.A. Noujaim for their supervision, encouragement and support. I would like to thank Miss Laura Lam for her help in revising my thesis. I also like to thank my colleges F.T. Kreutz, D.Z. Xu and F. Lui for their suggestions in my studies.

Table of Contents

CHAPTER 1 LITERATURE REVIEW 1

1. BISPECIFC ANTIBODIES.. 1
  1.1 Production of Bispecific Antibodies 2
    1.1.1 Chemically Coupling 3
    1.1.2 Somatic Hybridization 4
    1.1.3 Genetic Engineering.. 7
  1.2 APPLICATIONS OF BISPECIFIC ANTIBODIES 9
    1.2.1 Immunohistochemistry and Enzyme Immunoassays 9
    1.2.2 Radioimaging and Radioimmunotherapy... 10
    1.2.3 Immunotherapy 12
    1.2.4 Other Applications... 16
2. LIPOSOME 17
  2.1 LIPOSOME MORPHOLOGY 17
  2.2 LIPOSOME AS DRUG CARRIER.. 18
    2.2.1 Factors Important for Drug delivery using Liposome.. 18
    2.2.2 Stealth Liposome.. 19
    2.2.3 Immunoliposome.. 20
    2.2.4 Biotinylated Liposomes 21
3. DEVELOPMENT AND APPLICATIONS OF BSMAB - RESEARCH GOALS 23
  3.1 OBJECTIVE...23
  3.2 HYPOTHESIS... 23
  3.3 SPECIFIC AIMS... ... 23

CHAPTER 2 DEVELOPMENT AND CHARACTERIZATION OF A MONOCLONAL ANTIBODY TO BIOTIN 31
1. INTRODUCTION.. 31
2. MATERIALS AND METHODS 32
  2.1 Antigen Preparation 32
  2.2 Intrasplenic Immunization 33
  2.3 Hybridoma Fusion..33
  2.4 Screening by Direct ELISA...34
  2.5 Isotyping of MAbs 35
  2.6 Determination of Relative Binding Efficiency 35
  2.7 Cloning of Hybridoma 36
  2.8 Ascites Production and Purification 36
  2.9 Determination of Dissociation Constant... 37
3. RESULTS AND DISCUSSION.. 37
  3.1 Preparation of Biotin-Carrier Conjugates 37
  3.2 Determination of Degree of Biotinylation 38
  3.3 Development of Hybridoma P54.1R.. 39
  3.4 Isotype of Anti-Biotin Antibody 40
  3.5 Relative Binding Efficiency of Anti-Biotin Antibody.................................. 41
  3.6 Preparation of Anti-Biotin Antibody 41

3.7 Dissociate Constant Determined by ELISA Method 42

CHAPTER 3 A RAPID NON-SELECTIVE METHOD TO GENERATE QUADROMAS BY MICROELECTROFUSION 50

1. INTRODUCTION 50
2. MATERIALS AND METHODS 51
2.1 Hybridoma Cell lines 51
2.2 Microelectrofusion 51
2.3 Bispecific MAb Enzyme Immunoassays 52
3. RESULTS AND DISCUSSION 52

CHAPTER 4 DEVELOPMENT OF BSMAB (ANTI-BIOTIN X ANTI-HRPO) AS A UNIVERSAL IMMUNOPROBE FOR DETECTING BIOTINYLATED MACROMOLECULES 63

1. INTRODUCTION 63
2. MATERIALS AND METHODS 64
2.1 PREPARATION OF BIOTINYLATED PROTEIN AND LIPSOME 64
2.2 DEVELOPMENT AND CHARACTERIZATION OF BSMAB 65
2.2.1 Microelectrofusion 65
2.2.2 BsMAb Bridge ELISA 65
2.2.3 Production and Dual Affinity Purification 65
2.2.4 Determination of Binding Efficiency 66
2.2.5 Background Noise Determination 66
2.3 APPLICATIONS OF P58.5R IN DIFFERENT BIOASSAY SYSTEMS 66
2.3.1 Immunoassay 66
2.3.2 Liposomal Immunosorbent Assay 67
2.3.3 Quantitative Measurement of Biotin 67
2.3.4 Dot Blot and Western Blot 68
3. RESULTS AND DISCUSSION 68
3.1 Development of BsMAb P58.5R 68
3.2 Purification and Characterization of BsMAb P58.5R 69
3.3 Applications of BsMAb P58.5R in Different Bioassay Systems 71
3.3.1 Immunoassay of Biotinylated Proteins 71
3.3.2 Liposomal Immunosorbent Assay. 71
3.3.3 Quantitative Measurement of Biotin 72
3.3.4 Western Blot and Dot Blots 72
3.3.5 Detection of Biotinylated DNA 72
3.3.6 Other Applications 73

CHAPTER 5 A SIMPLE AND EFFICIENT METHOD FOR RADIOLABELING OF PREFORMED LIPOSOMES 86

1.INTRODUCTION 86
2.MATERIALS AND METHODS 87
2.1 Liposome Preparation 87
2.2 Labeling Procedures 88
2.3 Chloroform Extraction 88

  2.4 Stability of ^{99m}Tc Labeled Liposomes 89
  2.5 Biodistribution Studies 89
3. RESULTS AND DISCUSSION 89

CHAPTER 6 ENHANCED TUMOR SPECIFIC DRUG DELIVERY BY PRETARGETING BISPECIFIC MAB AND BIOTIN PEGYLATED LIPOSOME 100

1. INTRODUCTION 100
2. MATERIALS AND METHODS 102
  2.1 Preparation of BsMAb P60 (174H.64 x anti-biotin) 102
  2.1 BsMAb Enzyme Immunoassay for Selecting Quadroma 102
  2.3 Production and Purification 102
  2.4 Preparation of Biotin-Pegylated Liposome and Biotin Conventional Liposome 103
  2.5 Sandwich Immunoassay for Detecting Biotin Moiety of Biotin Pegylated Liposome. 104
  2.6 Agglutination Studies by Streptavidin 104
  2.7 Relative Avidity of BsMAb with D-Biotin and Biotin-Pegylated Liposome 104
  2.8 Loading of Model Drug ^{99m}Tc –DTPA Into Biotin-Pegylated Liposome 105
  2.9 Stability of Liposome Loaded with ^{99m}Tc-DTPA In Vitro 105
  2.10 Targeting of Biotin Pegylated Liposome to KLN-205 cells by BsMAb *in vitro* 106
  2.11 Biodistribution Studies on Mice Bearing KLN-205 Allografts 106
  2.12 Effect of Administration of Streptavidin *in vivo* to Reduce the Blood Background.. 107
3. RESULTS AND DISCUSSION 107
  3.1 Preparation of BsMAb P60 (174H.64 x anti-biotin) 107
  3.2 Preparation of Biotin Pegylated Liposome 108
  3.3 Establishment of Specific Drug Delivery System 111

SUMMARY AND FUTURE WORKS 122

REFERENCES 127

LIST OF FIGURES

Figure 1.1 A schematic representation of a variety of bsMAbs. 25

Figure 1.2 Production of quadromas. 26

Figure 1.3 Schematic representation of different applications of bsMAb in therapeutics. 27

Figure 1.4 Morphology of liposomes and lipid bilayer construction. 28

Figure 1.5 Schematic representation of stealth-liposomes and conventional liposomes. 29

Figure 1.6 Antibody-liposome conjugates used as targeting reagents for detection or therapeutic applications. 30

Figure 2.1 Structure of biotin (MW 244.31 D). 43

Figure 2.2 Schematic representation of conventional hybridoma technology. 44

Figure 2.3 Relative affinities of anti-biotin antibodies for biotinylated BSA determined by a residual titration immunoassay. 45

Figure 2.4 Scatchard plot of monospecific MAb P54.1R against biotin. 46

Figure 3.1 Diagrammatic representation of BTX meander chamber. 59

Figure 4.1 Diagrammatic representation of sandwich immunoassay for detecting biotin moieties of biotin pegylated liposomes. 75

Figure 4.2 Schematic representation of dual affinity chromatography. 76

Figure 4.3 Purification of bsMAb P58 using biotin-agarose affinity column. 77

Figure 4.4 Relative binding efficiency of bsMAb vs. streptavidin-HRPO for biotinylated OVA. 78

Figure 4.5 Effect of antigen-antibody reaction time and color developing time on the bsMAb ELISA. 79

Figure 4.6 The limit of detection of biotin by bsMAb P58.5R in ELISA. 80

Figure 4.7 Sandwich immunoassay for detecting biotinylated liposome with varying PEG concentration. 81

Figure 4.8 Determination of biotin concentration with competitive ELISA method. 82
Figure 4.9 West Blot using bsMAb as the Detection Reagent. 83
Figure 4.10 Dot Blot using bsMAb as the Detection Reagent. 84
Figure 5.1 Structure of ^{99m}Tc-DISIDA complex (MW. 894 D). 94
Figure 5.2 Sephadex G-25 column separation profile of ^{99m}Tc labeled liposomes and ^{99m}Tc-DISIDA alone. 95
Figure 5.3 Retention of radioactivity in liposomes from dialysis of ^{99m}Tc labeled liposomes as a function of incubation time at 37 °C. 96
Figure 5.4 Biodistribution curve shows the difference of tissue uptake between ^{99m}Tc labeled liposomes and free ^{99m}Tc-DISDIA. 97
Figure 6.1 Schematic representation of strategy for anti-cancer drug delivery. 114
Figure 6.2 Relative binding avidity of bsMAb (174H.64 X anti-biotin) with biotin and biotin pegylated liposome. 115
Figure 6.3 Effect of biotin content on the agglutination of biotin pegylated liposome induced by streptavidin. 116
Figure 6.4 Stability of ^{99m}Tc-DTPA loaded biotin pegylated liposome incubated with normal saline and 100% FBS at 37^{0}C for 24 h. 117
Figure 6.5 Targeting of biotin pegylated liposome loaded with ^{99m}Tc-DTPA to KLN-205 cells by bsMAb (174H.64 X anti-biotin) *in vitro*. 118
Figure 6.6 Biodistribution on different post-injection time. 119
Figure 6.7 Effect of streptavidin on the tissue biodistribution. 120

LIST OF TABLES

Table 2.1 Immunization Protocol for Generation of Mab against Biotin. 47

Table 2.2 Screening of Primary Hybridomas for Anti-biotin Reactivity by ELISA 48

Table 2.3 Isotype of Anti-biotin Antibodies from P54 clones. 49

Table 3.1 Screening Methods Employed for Identifying Various Quadromas Developed by Microelectrofusion. 60

Table 3.2 Primary Screening of Quadromas Secreting Bispecific Antibodies. 61

Table 3.3 Summary of Results of the Different Microelectrofusion Experiments Generating Quadromas. 62

Table 4.1 Specific Binding Background Noise with Various Biotin Binding Macromolecules. 85

Table 5.1 Chloroform Extraction of Radioactivity from the Saline Phase of Different Formulations. 98

Table 5.2 Tissue Biodistribution of ^{99m}Tc Labeled Liposomes at 24 H Post-Injection 99

Table 6.1 Results of Tissue Biodistribution of ^{99m}Tc-DTPA Loaded Liposomes at 6 H Post-Injection with Pretargeted BsMAb. 121

LIST OF ABBREVIATIONS

A280nm	Absorbance at 280 nm
ABTS	2,2'-Azino-di [3-ethylbenzthiazolin sulfonate]
Ab	Antibody
AC	Alternative current
Ag	Antigen
APCs	Antigen-presenting cells
B-PE	Biotin-phosphatidylethanolamine
BSA	Bovine serum albumin
bsMAb	Bispecific monoclonal antibody
CH	Cholesterol
C_H	Immunoglobulin heavy chain constant domain
C_L	Immunoglobulin light chain constant domain
CTL	Cytotoxic T lymphocytes
Da	Daltons, unit of molecular weight
DC	Direct current
DISIDA	Diisopropyl iminodiacetic acid
DSPC	Distearoyl-phosphatidylcholine
DSPE	Distearoyl phosphatidylethanolamine
DTPA	Diethylenetriaminepentaacetic Acid
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
Fab	Fragment of antibody antigen binding site
FACS	Fluorescence-activated cell sorter
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate

g	Gravity
h	Hour(s)
HABA	4-hydroxy-azobenzene-2'-carboxylic acid
HAT	Hypoxanthine, Aminopterin and thymidine
HRPO	Horseradish peroxidase
HSPC	Hydrogenated soy phosphatidylcholine;
HMPAO	Hexamethylpropyleneamine oxime
i.e.	For example
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
i.p.	Intraperitoneal
i.v.	Intravenous
IRMA	Immunoradiometric assay
KLH	Keyhole limpet haemocyanin
L	Liter(s)
LLD	Lowest limit of detection
M	Molar
MAb(s)	Monoclonal antibody (antibodies)
min	Minute(s)
MLV	Multilamellar vesicles
mm	Millimeter
mM	Millimolar
MW	Molecular weight
NHS-biotin	N-Hydroxysuccinimide ester biotinamidocaproate
OD	Optical density
OPI	Oxaloacetate, pyruvate and insulin
OVA	Ovalbumin

ng	Nanogram(s)
nm	Nanometer(s)
nmole	Nanomole(s)
OD	Optical density
OVA	Ovalbumin
P54	Hybridoma secreting MAb against biotin
P58	Quadroma secreting bsMAb anti-biotin x anti-HRPO
P60	Quadroma secreting bsMAb anti-biotin x anti-squamous carcinoma
PBS	Phosphate buffered saline pH 7.4
PE	Phosphatidylethanolamine
PEG	Polyethylene glycol.
s.c.	Subcutaneous
ScFv	Single chain antibody variable region
SD	Standard deviation
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
TCR	T cell receptor
TRITC	Tetramethylrhodamine isothiocyanate
V_H	Immunoglobulin heavy chain variable domain
V_L	Immunoglobulin light chain variable domain
v	Volume
w	Weight

CHAPTER 1

LITERATURE REVIEW

Part of this chapter has been published by Bioconjugate Chemistry 9, 635-644, 1998.

To completely control cancers and cardiovascular diseases, the major causes of morbidity in the Western world, developing efficient targeting systems is a pressing need. To improve life quality of elderly, with the emergence of an aging population, selective targeting for treatments of CNS related diseases is demanding. To reduce patient's resistance to commonly used antibiotics, improved targeting chemotherapy is an imminent need. To understand and cure viral diseases including AIDS, targeted therapy will definitely play an invaluable role.

Drug targeting through carrier system has been a central topic of research in biomedical areas. Several carriers have been explored to deliver drugs toward the specific targets. Commonly used drug carriers include: liposomes, antibodies, albumin conjugates, dextran, lectin etc. (Vemuri and Rhodes, 1995). Targeting could undoubtedly play an important role in concentrating drugs locally while minimizing toxicity.

1. BISPECIFC ANTIBODIES

Monospecific antibodies exemplified by the naturally occurring major IgG class have two identical antigen binding paratopes due to their make-up with two identical heavy and light chains. The first bispecific polyclonal antibodies were generated using chemical methods by Nisonoff and Rivers in 1961 (Nisonoff and Rivers, 1961). However, the concept of bispecific monoclonal antibodies with two different specifications was introduced by Milstein and Cuello in 1983 (Milstein and Cuello, 1983). In the past two decades, hybridoma technology established itself as one of the cornerstones of modern biotechnology (Kohler and Milstein, 1975). As a result, bsMAb represent second generation of monoclonals developed for a variety of *in vivo* and in vitro uses.

A number of applications for MAbs involve chemical manipulation of the antibody to create covalent immunoconjugates with radionuclides, enzymes, toxins, drugs and a variety of other haptens. Early chemical conjugation methods using homobifunctional cross-linkers generated complex aggregates of the two entities. Refinement in chemical conjugation, employing heterobifunctional cross-linkers, has mitigated the problems but not entirely eliminated them (Hermansen, 1996). Bispecific antibodies with intrinsic binding sites to any two antigens have the capability to form uniform, homogeneous and reproducible immunoconjugates with one or two entities in a predetermined order (Suresh *et al.*, 1986a). As such binding is non-covalent and dependent largely on the affinity of the epitope-paratope interaction, bsMAbs have a singular advantage of being able to cross-link two cells for targeting purpose *in vivo* (Staerz and Bevan, 1989). The latter feature has not been described to date using chemical conjugation methods for *in vivo* applications. Current applications of bsMAbs range from immunohistochemistry (Milstein and Cuello, 1983), immunoassays (Kreutz and Suresh, 1995 and 1997; Suresh *et al.*, 1986a), complement-mediated cytotoxicity (Wong and Colvin, 1987) and *in vivo* retargeting (Fanger *et al.*, 1995; Demanet *et al.*, 1996; Somasundaram *et al.*, 1996).

The bsMAb has great potential in a wide variety of research and eventually, clinical uses. Some of these exciting explorations include redirecting cytotoxicity to tumor cells or virus, developing antibody-directed enzyme prodrug therapy (ADEPT), targeting fibrinogen activator to dissolve fibrin clots and generating effective vaccines.

1.1. Production of Bispecific Antibodies

Many different types of bsMAbs have been made (Fig 1.1, p 25) by one of three general methods: (1) chemically cross-linking two antibody molecules or antibody fragments, (2) fusion of two different cell lines to form a quadroma or trioma, (3) recombinant DNA based approaches.

1.1.1 Chemically Coupling

More than 30 years ago, the first polyclonal antibodies with two different specifications were produced by chemically coupling two different polyclonal antibodies (Nisonoff and Rivers, 1961). This chemical manipulation involved the dissociation of the two different antibodies, mixing and reassociation of the two half molecules. A portion of the reassembled antibodies was shown to be bispecific in nature by immunohistochemistry. With the advent of monoclonal antibodies (MAb) and improvements in the chemistry of preferential formation of heteroconjugates, developing bispecific monoclonals became a reality, eliminating the heterogeneous polyclonal reagents.

A large number of chemical reagents have been used for selectively cross-linking proteins through specific functional groups. Bifunctional reagents reactive with ε-amino groups or with hinge region thiol groups have been most useful in preparing bsMAb and other types of immunoconjugates. These cross-linkers can be classified into two categories, homo and heterobifunctional reagents.

The homobifunctional reagents react with the free thiol groups generated by reducing inter heavy chain disulfide bonds. 5, 5'-dithiobis(2-nitrobenzoic acid) (DTNB) or o-phenylenedimaleimide (O-PDM) as a maleimide-containing compound can activate thiols on Fab' fragments of MAb (Brenner *et al.*, 1985; Glennie *et al.*, 1987; Shalaby *et al.*, 1992). DTNB acts to regenerate disulfide bonds between the two Fab's, whereas O-PDM acts to form a thioether bond between the two Fab'. Generally, the thioether bond is more stable than the disulfide bond regenerated (Glennie *et al.*, 1987). In addition to dimeric bsMAbs, trimeric and tetrameric antibodies with two or three specificities have been constructed using O-PDM and shown to have more potent redirecting effector cell tumor cytotoxicity compared to the corresponding dimeric antibodies (Tutt *et al.*, 1991a and b).

The heterobifunctional reagents can introduce a reactive group into a protein enabling it to react with a second protein. N-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP) can react with primary amino groups to introduce free thiol groups

(Jung *et al.*, 1991; Segal *et al.*, 1988). As a result, SPDP can combine any two proteins that have exposed amino groups including antibodies and Fab' fragments, regardless of class or isotype (Van Dijk *et al.*, 1989). Unfortunately, this approach causes random cross-linking of the molecules and hence exhibits batch to batch variations.

The improvements in cross-linking technology have led at least one biotechnology company (Medarex Inc., NJ) to develop bispecific (Fab')$_2$ antibodies and evaluate their utility in FDA approved human clinical trials (Valone, *et al.*, 1995). Chemical linking that has been highly refined is more straightforward, faster, higher yields, and the products are comparatively easier to purify (Glennie *et al.*, 1987). However, denaturation of antibody is a concern during these types of chemical reactions.

1.1.2 Somatic Hybridization

Somatic hybridization for generating hybrid antibodies appeared as early as 1973 (Cotton and Milstein, 1973), although the term and concept of bispecific antibodies were not appreciated. Basically, somatic hybridization included fusion of two hybridomas and purification of the bsMAb generated by the resulting quadromas. Two general methods have been introduced to generate hybrid-hybridomas secreting bsMAb: (1) fusion of one established hybridoma with lymphocytes derived from a mouse immunized with a second antigen generates trioma (Milstein and Cuello, 1983), (2) fusion of two established hybridomas generates a quadroma (Suresh *et al.*, 1986b).

Somatic hybridization for development of bsMAb involves methods similar to those for preparing hybridomas (Kohler and Milstein, 1975). It has been greatly facilitated by the use of drug resistance markers introduced into cell lines by conventional and molecular genetic techniques. First, each hybridoma cell line is made resistant to a specific drug-resistant marker. Quadroma cells are then selected after fusion using a culture medium containing both drugs which will kill both parental cells allowing only quadroma to survive (Suresh *et al.*, 1986b; De Lau *et al.*, 1989). Alternatively a universal fusion partner could be made by sequentially culturing in 8-azaguanine and ouabain to generate a HAT sensitive and ouabain resistant cell line (Fig 1.2, p 26). This universal fusion partner can then be fused with a second normal hybridoma which is HAT resistant and ouabain sensitive. Only the fused quadromas can survive in the combined HAT and ouabain selection medium.

The advantage of using drug resistance markers is that the selected fusion cells are functionally stable synkarions (fused nuclei). Many techniques are available to confer a specific drug resistance marker to a hybridoma cell line. Selection of spontaneous mutants acquiring resistance to a certain drug is commonly used. Azaguanine or bromodeoxyuridine can be used to select mutants with deficiencies in the purine and pyrimidine salvage pathway. This selection is based on mutations in the genes coding for hypoxanthine guanine phosphoribosyltransferase (HGPRT) or thymidine kinase (TK) (Milstein and Cuello, 1983; Tada *et al.*, 1989; Urnovitz *et al.*, 1988; Wong and Colvin, 1987). These mutations render the cell sensitive to aminopterin, one of the components of HAT (hypoxanthine, aminopterin and thymidine) medium. Other drugs used are ouabain, emetine, actinomycin D, methotrexate, iodoacetamide and neomycin (De Lau *et al.*, 1989; Staerz and Bevan, 1986; Suresh *et al.*, 1986a and b; Tiebout *et al.*, 1987).

The isolation of spontaneous drug resistance mutants can be a time-consuming process due to a low frequency of mutation, and sometimes can cause the loss of antibody production (Nolan and Kennedy, 1990). Alternative methods are to introduce a bacterium-derived marker-gene coding for a dominant drug resistant marker. Due to the high transfection frequency, a retroviral shuttle system has been used to introduce the gene coding for aminoglycoside phosphotransferases, acquiring resistant to the neomycin analogue G418-sulphate (Demonte *et al.*, 1991). Considering a risk of activation of retrovirus, electroporation is another choice to transfect the hybridomas safely by a bacterial plasmid. It is still difficult to clearly understand the mechanisms involved in the stable integration and expression of the bacterial gene in the eukaryotic genome after electroporation. However, recent developments in the methodology have led to an efficient and stable transfection of a hybridoma cell line (Bos and Nieuwenhuizen, 1992).

Somatic hybrids can also be selected by sorting out the heterofluorescent cells without relying on drug selection (Karawajew *et al.*, 1987; Karawajew *et al.*, 1988). Here, hybridomas were first pre-labeled with either fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC) *via* the cell membrane marker octadecylamine. Differentially labeled hybridomas are then fused and the cells stained by both fluochromes are isolated using a fluorescence-activated cell sorter (FACS). Electrofusion plus FACS selection may further increase yields of quadromas (Kreutz *et al.*, 1998).

BsMAb activities from putative quadroma clones should be tested as quickly as possible. A bridge ELISA is a general way to screen bsMAb (DeLau *et al.*, 1989; Koolwijk *et al.*, 1988; Suresh *et al.*, 1986a). Double antigen ELISA may be employed provided that sufficient quantities of the respective antigens are available. The first antigen coated ELISA plates are incubated with supernatant, and antibody reactivity to the second antigen is performed using antigen labeled with an enzyme marker. Alternatively, when two different H chain isotypes are present in the bsMAb, isotype specific reagents could be used for detection of hybrid molecules. BsMAb is captured by antigen or antibody against one IgG subclass coated on ELISA plate and another isotype is used to be detected in an ELISA format. Lastly, the potential of bsMAb to redirect T cell cytotoxicity could be tested utilizing cytotoxic T cell clones and targeted cell lines (Haagen *et al.*, 1992).

Biologically produced bsMAb are synthesized, assembled and secreted by the same process as native immunoglobulins, and thus they have similar characteristics in terms of stability and pharmacokinetics (Songsivilai and Lachmann, 1990). Since individual light chain and heavy chain are transcribed separately and are assembled randomly, the desired bsMAb produced by the quadroma could range from 10-50% (Suresh *et al.*, 1986b). Purification of this bsMAb is essential and has been achieved by hydroxylapatite chromatography (De Lau *et al.*, 1992; Karawajew *et al.*, 1987), ion-exchange chromatography (Auriol *et al.*, 1994; Suresh *et al.*, 1986b), double affinity chromatography (Sahin *et al.,* 1990; Smith *et al.,* 1992), mimetic affinity methods (Xu, *et al.,* 1998) and gradient thiophilic T-gel chromatography (Kreutz, *et al.,* 1998).

1.1.3 Genetic Engineering

Production by the chemical conjugation or cell fusion generates a heterogeneous mixture of different products, requiring labor intensive methods to purify the bispecifc form from the other homo and hetero aggregated species. Many of the limitations of generating bsMAb through these traditional methods may be potentially overcome by genetic engineering techniques. This new technique was first described by Songsivilai and colleagues to generate transfectants for secreting humanized bsMAb (Songsivilai *et al.,* 1989). The common method is to express antibody or its fragment of different specificities as distinct polypeptides from the same transcript or from separate plasmid constructs. Subsequently, the different antibody fragments then combine through binding motif (Shalaby *et al.,* 1992). Recently, bsMAb molecules have been made as a single covalent structure by combining two single chain Fv fragments using polypeptide linker (Holliger *et al.,* 1993; Kranz *et al.,* 1995; Schmidt *et al.,* 1996; Thirion *et al.,* 1996; Winter and Milstein, 1991). Different spacers have been used to increase the bispecific yield (Gruber *et al.,* 1994; Mallender *et al.,* 1994). It is essential in this strategy to ensure that the two single chain antibodies exhibit affinities similar to the parental MAbs.

Ridgway and associates reported a novel "knobs into holes" method to make bsMAb (Ridgway *et al.,* 1996). Here, the knobs were created by replacing small amino acid side chains at the interface between C_H3 domains with larger ones, whereas the holes were generated by replacing large side chains with smaller ones. This engineering of the C_H3 domains of heavy chains resulted in the formation of heterodimers (Ab1-Ab2) more favorable than homodimerization between Ab1-Ab1 and Ab2-Ab2.

Holliger and his colleagues developed a diabody method to generate bsMAb fragments (Holliger *et al.,* 1993). The diabody method constructs scFv chains with short linkers, which prevents intrachain pairing but allows interchain pairing to form bivalent fragments. If the scFvs contain V_L and V_H for two different antigen targets, then assembly with the reciprocal scFv pair can generate bsMAb fragments. Moreover, a phage display diabody approach was developed to generate and screen bispecific scFvs (McGuinness, *et al.*, 1996). Although the diabody approach appears promising with previously cloned scFv molecules, construction of diabody libraries from large Ab repertoires requires multiple steps. The stability of the library in bacteria has not yet been extensively evaluated (Hayden, *et al.*, 1997).

Leucine zipper-based dimerization of MAb fragments has also been introduced recently (Kostelny *et al.*, 1992). The leucine zipper are sequences derived from regions of the transcription factors *fos* and *jun*. A murine anti-CD3 Fab'-*fos* and anti-Tac Fab'-*jun* were individually expressed as homodimers in SP2/0 cells. When reduced, mixed and reoxidized, a preferential heterodimer association, anti-CD3 x anti-Tac, was formed due to the formation of a *fos* and *jun* leucine zipper. De Kruif and Logtenberg also described the fusion of *fos* or *jun* leucine zipper and a truncated mouse IgG3 hinge region to scFv proteins (deKruif and Logtenberg, 1996). In their method, two cysteine residues were engineered into the zipper domains to produce disulfide-stabilized homodimers. Using scFvs isolated from a phage display library and these modified zipper cassettes, they have produced functional stable secreted homodimers in *E. coli*. After further modification of the homodimers, functional $bs(scFv)_2$ molecules were developed. Rheinnecker also utilized a similar approach to create scFv dimers by fusion of an artificial helix-turn-helix dimerization domain of p53 to a long IgG3 hinge-scFv construct (Rheinnecker, *et al.*, 1996).

Among these several of approaches to genetically engineer bispecific antibodies, the most promising approach appears to be the single chain bispecific Fv and bispecific diabodies. This is due to their small size and hence the more attractive for *in vivo* applications exhibiting faster clearance, better tissue penetration and decreased immogenecities.

1.2 APPLICATIONS OF BISPECIFIC ANTIBODIES

1.2.1 Immunohistochemistry and Enzyme Immunoassays

The advent of MAb technology was instrumental in the development and refinement of immunoassays using chemically linked radioisotopes, enzymes, fluorochromes or others detection moieties. Despite the surge in these new applications, chemical cross-linking has many disadvantages: 1) Chemical cross-linking is random generally resulting in batch to batch variations in reagent quality. 2) The size of conjugates or complexes obtained are not uniform thus influencing their penetration in immunohistochemical applications. 3) Active site or binding site inactivation of markers and/or immunoglobulins may be encountered. 4) Most often irreversible covalent linkages are formed which are advantageous for in vitro applications but are less suitable for *in vivo* therapeutic studies. These drawbacks of covalent cross-linking of antibodies to enzyme or radioactive markers often influence the linearity and reproducibility of the assay method. BsMAbs designed to specifically cross-link the antigen non-covalently with a signal generating marker such as an enzyme eliminates the need for chemical conjugation, generating a greater degree of linearity, sensitivity and reproducibility (Suresh, 1991).

Milstein and Cuello (Milstein and Cuello, 1983) first developed a bsMAb, anti-somatostatin X anti-peroxidase, which was used in immunohistochemistry. Suresh and his co-workers (Suresh *et al.*, 1986a) established a quadroma secreting anti-peroxidase X anti-substance P, which simplified the immunohistochemical procedures and gave a better sensitivity. A bsMAb reacting with both horseradish peroxidase (HRPO) and fluorescein isothiocyanate (FITC) was used to label different MAbs in EIA systems (Karawajew *et al.*, 1988). Many investigators have developed bsMAbs directed against enzyme (e.g. HRPO, alkaline phosphatase and β-galactosidase) and a second antigen (e.g. tumor specific antigen, peptide or hormone) for use in EIA or immunohistochemistry (Allard *et al.*, 1992; Auriol *et al.*, 1994; Berkova *et al.*, 1996; Jantscheff *et al.*, 1993; Kontsekova *et al.*, 1992; Kreutz and Suresh, 1995 and 1997; Liu *et al.*, 1995; Piran *et al.*, 1993; Ribeiro-da-Silva *et al.*, 1991;

Tada *et al.*, 1989; Takahashi and Fuller, 1988; Wognum *et al.*, 1989). Rylatt developed a homogeneous immunoassay system to detect circulating antigens in whole blood. The bsMAb used here cross-linked specific antigen of interest and red blood cells, causing agglutination of a patient's own blood sample (Rylatt *et al.*, 1990).

1.2.2 Radioimaging and Radioimmunotherapy

Conventional radioimmunoimaging and in particular radioimmunotherapy with MAb conjugates is severely limited by radiation toxicity to normal organs, especially blood, liver, kidney and bone marrow. This is largely due to the high radiation dose from prolonged circulating radiolabeled antibody to these organs, from normal tissue accumulation of the antibody, or from free radionuclide deposited in these organs. Pretargeted imaging and radioimmunotherapy with bsMAb is a two or three step strategy for delivering radioisotope (^{99m}Tc, ^{90}Y, ^{67}Ga, ^{111}In) to a tumor quickly and specifically with minimal radiation exposure and hence lowers the dose to normal organs (Chetanneau *et al.*, 1994; Chatal *et al.*, 1995; Dillehay *et al.*, 1995; Kranenborg *et al.*, 1995; Schuhmacher *et al.*, 1995).

Pretargeting involves administration of a bsMAb having a high affinity binding site for a small rapidly excreted effector molecule, which is given as a second step after the bsMAb has concentrated in the target tumor. Removal of the excess bsMAbs from the circulation with a clearing agent is an optional step before giving the effector molecule for greatly improved target-to-blood ratio. Soon after the chase, the effector molecule is given, and the maximum tumor concentration and tumor-to-normal tissue ratio is achieved in 1-3 h. Unbound radiolabel (more than 90% of injected dose) is rapidly excreted *via* the kidney, by glomerular filtration mechanism, leading to greatly decreased radiation exposure to normal tissue. In contrast, about 95% of ^{90}Y-MAb monospecific conjugates are retained at 3 h and 85% at 24 h (Goodwin *et al.*, 1994). Several different targeting macromolecule and effector small molecule pairs have been proposed in the literature (Goodwin, 1987).

Pretargeting combines the pharmacokinetics of long circulating MAbs with rapidly excreted small effector molecules to give both high tumor concentration and high tumor-to-normal tissue ratios (Goodwin *et al.,* 1994). The two or three step methods eliminate radiation exposure during the MAb localizing phase, which can take up to several days. Nonspecific localization at this stage in liver, spleen and bone marrow does not contribute to normal tissue radiation since the radioactive hapten is injected later. Previous attempts to overcome this problem have required the administration of large amounts of cold MAb along with directly labeled MAb to saturate the nonspecific binding sites. The total radiation dose to normal tissue is greatly reduced in the pretargeting method by two mechanisms: (1) absence of radiation during the localization phase, since the bsMAb is not radioactive at this time; (2) rapid renal excretion of excess radiolabeled hapten that is not bound to the bsMAb in the tumor. An obvious limitation of this two or three step pre-targeting strategy is that bsMAbs directed to rapidly internalizing antigens would fail to capture and localize the radiolabeled hapten. Hence, bsMAbs which bind and are poorly internalized are most useful in this strategy.

One bifunctional antibody currently under clinical evaluation was made by the chemical coupling of a Fab' fragment of an anti-carcinoembryonic antigen (CEA) antibody to a Fab' fragment of an anti-DTPA-indium antibody. In patients with colorectal or medullary thyroid cancer, clinical studies performed with this anti-CEA/anti-DTPA-indium bifunctional antibody and an indium-111-labeled bis-DTPA bivalent hapten showed that tumor uptake was not altered compared to the results for $F(ab')_2$ fragments of the same anti-CEA antibody directly labeled with indium-111, whereas the radioactivity of normal tissues was significantly reduced (3 to 6-fold). The fast tumor uptake kinetics and high tumor-to-normal tissue ratios obtained with bifunctional antibody techniques exhibit parameters favorable for efficient radioimmunotherapy (Chatal *et al.,* 1995).

Pretargeting methods have also incorporated the use of biotin or avidin labeled MAb followed by radiolabeled avidin or biotin respectively (Van Osdol *et al.,* 1993). In this approach, however, the immunogenecity of the chicken egg avidin or streptavidin could be a potential limitation. An additional limitation of this strategy is that the requirement of 2-3 agents places an additional manufacturing burden and significantly complicates the design of clinical trials.

1.2.3 Immunotherapy

BsMAbs are capable of activating and targeting the cellular immune defense system to kill tumor cells or other pathogens (Fig 1.3, p 27). Many groups have already demonstrated lysis of target cells by T effector cells using bsMAb in a non-MHC (major histocompatibility complex) restricted fashion. (Canevari *et al.*, 1988; Roosnek and Lanzavecchia, 1989; Roosnek *et al.*, 1990). The primary cytotoxic trigger complex on T cells is the TcR complex, which consists of a noncovalent association of Ti heterodimers with the CD3 molecular complex. The TcR is normally antigen specific and MHC restricted. However, a bsMAb can react with this complex and initiate targeted cytotoxicity by activating the T-cell in a manner that is not MHC restricted. A bsMAb against both a tumor and the CD3 complex can thus redirect the cytolytic action of virtually all available T cells to the antigen of choice. This *in vivo* cross-linking of specific targets with components of the cell-mediated immune response such as T-cells, natural killer cells, macrophages, dendritic cells and large granular lymphocytes is a unique feature outside the realm of chemical cross-linking methods.

Many studies of cytotoxicity have involved binding of MAb to these surface molecules such as the TcR heterodimer (Staerz and Bevan, 1985), the CD3 complex (Liu *et al.*, 1985; Rammensee *et al.*, 1987) and the CD2 molecule (Bolhuis *et al.*, 1991; Tutt *et al.*, 1991b). This activation process and the triggering of cell proliferation, lymphokine production, and cytotoxicity have gained great interest since it has been hypothesized that sufficient number of cytolytic T cells could be localized specifically to the tumor cells. Many efforts have been made to use bsMAb to focus cytotoxic effector-cell response to tumor targets. Cytotoxicity is not due to bystander lysis, since direct contact between effector and target cells is required (Barr *et al.*, 1987; Lanzavecchia and Scheidegger, 1987).

Cancer immunotherapeutic strategies involving bsMAb mediated killing have been explored by several groups including: anti-epidermal growth factor receptor for breast cancer (Knuth *et al.*, 1994), anti-sialyl Lewis A for colon cancer (Ohta *et al.*, 1995), anti-ovarian cancer (Bolhuis *et al.*, 1992; Canevari *et al.*, 1995), anti-renal cell carcinoma (van Dijk *et al.*, 1991), anti-lung small cell carcinomas (Azuma *et al.*, 1992), anti-CD19 for B lymphoma (Anderson *et al.*, 1992; De Gast *et al.*, 1995), anti-CD13 for acute myeloid leukemia (Kaneko *et al.*, 1993) and anti-tenascin for gliomas (Davico Bonino *et al.*, 1995). The therapeutic success of bsMAb was mainly based on the retargeting of the cytotoxic T cell which was further augmented by anti-CD28 co-stimulation (Demanet *et al.*, 1996). Other types of effector molecules which have been used are FcγRIII receptor (CD16) (Hsieh-Ma *et al.*, 1992; Weiner *et al.*, 1995), complement receptor CR3 of macrophage (Somasundaram *et al.*, 1996), high-affinity Fc receptor (FcγRI) (Deramoudt *et al.*, 1992) and anti-gamma/delta T cell receptor (Ferrini *et al.*, 1989).

The exciting pre-clinical studies have prompted several clinical studies using bsMAbs. The toxicities associated with this immunotherapeutic approach appears to be minimal and early results show promising clinical benefit as well. Guyre and Franger have developed a bsMAb, MDX-210, which recognizes FcγR1 on monocytes and macrophages and the cell surface product of the HER-2/neu oncogene, which is over-expressed on some breast and ovarian cancers. Clinical trials have demonstrated that treatment with MDX-210 is well tolerated and that MDX-210 is both immunologically and clinically active. Optimization of the dose and schedule of MDX-210 and development of combination treatments with cytokines that modulate immune effector cells will greatly enhance the efficacy of this novel bsMAb construct for treatment of tumors that over-express HER-2/neu (Valone *et al.*, 1995).

Another bsMAb 2B1 with specificity for the c-erbB-2 and FcγRIII extracellular domains has been tested in humans. This bsMAb promotes the targeted lysis of malignant cells over-expressing the c-erbB-2 gene product of the HER2/neu proto-oncogene by human natural killer cells and mononuclear phagocytes expressing the FcγRIII A isoform. In a Phase I clinical trial, the 2B1 treatment induced more than

100-fold increases in circulating levels of tumor necrosis factor-alpha, interleukin 6, and interleukin 8 and lesser rises in granulocyte-monocyte colony-stimulating factor and IFN-gamma (Weiner, *et al.*, 1995).

BsMAbs have also been tested for their utility in immunotherapeutic applications in viral infections (Berg *et al.*, 1991). Chamow and his associates have developed a humanized, bispecific antibody, comprised of CD4-IgG and humanized anti-CD3-IgG, that targets and kills HIV-infected cells. In targeting it exploits the natural affinity of CD4 for gp120 to target the bsMAb to HIV-infected cells. It then recruits and activates, through its anti-CD3 moiety, cytotoxic T lymphocytes (CTL) to lyse target cells in a non-MHC restricted manner. The ability of purified bsMAbs to specifically lyse HIV-infected target cells was demonstrated using CTL from two different sources: whole peripheral blood lymphocyte (PBL) fractions and pure CTL preparations. In contrast, a human anti-gp120 monospecific antibody mediated lysis of HIV-infected target cells only with PBL fractions and not with purified CTL. Moreover, lysis observed in the presence of the human anti-gp120 antibody was completely blocked in the presence of human serum (which competes for Fc gamma receptor binding with abundant human IgG), whereas bsMAb-mediated lysis of target cells was not affected. The bsMAb based lytic activity in the presence of serum reflects its unique ability to recruit CTL as effector cells and highlights a potentially important advantage of this type of construct over monospecific antibodies for HIV-directed therapy. (Chamow *et al.*, 1994 and 1995).

BsMAb could be also used as an effective drug delivery system for cancer treatment. BsMAbs have been made with one binding site to methotrexate (Affleck and Embleton, 1992), saporin (Bonardi *et al.*, 1992; Bonardi *et al.*, 1993; French *et al.*, 1995), doxorubicin (Reddy and Ford, 1993), or vinca alkaloids (Corvalan *et al.*, 1987). Clinical studies have been described using a combination of two bsMAbs to deliver the ribosome-inactivating protein, saporin, in the treatment of low-grade, end-stage, B-cell lymphoma. Two bsMAbs, each having one arm directed at saporin and one at the CD22 on target B cells. The bsMAbs, however, recognized different, non-overlapping epitopes on each molecule, a strategy which permitted high-avidity double attachment of saporin to the target. Five patients were treated, mostly with weekly doses of between 2 and 4 mg of saporin for a period of up to 6 weeks. Toxicity was minimal and all patients showed a rapid and beneficial response to treatment. When present, circulating tumor cells were cleared (4/4 patients), ascitic and pleural effusions were eliminated (2/2 patients) and one patient with splenomegaly showed a marked reduction in tumor bulk (French *et al.*, 1996).

Alternative strategies to antibody directed prodrug therapy include the development of bifunctional antibody enzyme fusion proteins. The advantage of the bsMAb plus enzyme and prodrug approach over fusion proteins is that the pharmacokinetics and target site accumulation of each component could be controlled separately. In addition, PEG-enzymes could be used for reduced immunogenicities.

BsMAbs can also be used to activate a prodrug *in vivo* to cause localized targeted cytotoxicity. In this approach, a bsMAb has been made against a tumor marker and a prodrug activating enzyme such as human placental alkaline phosphatase or *E.coli* β-lactamase. Activation of cephalosporin based anti-cancer prodrugs at the tumor site can be achieved with this bsMAb (De Sutter and Fiers, 1994). Another bsMAb against CD30 antigen and alkaline phosphatase was also used to activate the relatively nontoxic prodrug, mitomycin phosphate to mitomycin alcohol, which is 100-fold more toxic (Sahin *et al.*, 1990). The advantage of using bsMAbs to deliver toxins or drugs to tumors, compared to conjugating such moieties to monospecific MAbs is that chemical conjugations are totally avoided. This provides a more uniform cross-linking between the target and the effector molecule.

1.2.4 Other Applications

Tissue plasminogen activator (TPA) triggers the conversion of plasminogen to the fibrinolytic enzyme plasmin, which causes dissolution of thrombi. BsMAb can be used to improve fibrinolytic efficacy of TPA by binding simultaneously to human fibrin and TPA in thrombosis therapy (Kurokawa *et al.*, 1991). One study has shown that in the presence of TPA, bsMAb caused a 10-fold more fibrinolysis *in vitro* than with TPA alone (Branscomb *et al.*, 1990). Some researchers have also tried to use bsMAb to target urokinase-type plasminogen activator (UPA) to fibrin-containing clots (Charpie *et al.*, 1990). It caused 13-fold more fibrinolysis *in vitro* than did urokinase alone and 6-fold more clot lysis *in vivo*. In another study using baboon bearing ^{125}I-fibri clots in femoral vein, continuous intravenous infusion of a bsMAb resulted in a 5-fold enhanced thrombolytic potency of UPA over that of unconjugated UPA (Imura *et al.*, 1992).

BsMAbs could be generated to improve the binding specificity and avidity by recognizing two different adjacent epitopes in the same antigen. This type of a construct has been made using antigens such as human chorionic gonadotropic hormone (HCG), creatine kinase (CKMB) and adrenocorticotrophic hormone (ACTH) using bsMAbs against two different subunits of the hormone (Cheong *et al.*, 1990; Cook and Wood, 1994; Hakalahti *et al.*, 1993). A bsMAb against polyhedral boron anion and a tumor associated antigen, has been made to deliver a large amount of boron atoms to tumor cells for a novel radiotherapeutic application. Highly radiotoxic alpha particles are released from boron-10 when irradiated with low energy thermal neutrons to destroy tumors locally (Pak *et al.*, 1995).

The many applications of bsMAb as unique macromolecular heterobifunctional cross-linkers is expanding with promising prospects for clinical use as well. The sophistication of producing bsMAbs will grow hand-in-hand with the increased utility of these molecules as tools for research and as therapeutic agents. Recent advances in molecular biology and cancer research have added new dimensions to our investigations and understanding of how the bsMAb works and the novel applications where it has been employed.

2 LIPOSOMES

Liposomes are microparticulate lipoid systems consisting of one or more bilayers arranged concentrically with aqueous compartments and a core. They are made predominantly from naturally-occurring phospholipids and therefore are biodegradable, innocuous and do not cause immune response. Since they were introduced by Bangham (Bangham *et al.,* 1965), liposomes have been considered to be excellent models of cell membranes as well as useful carriers of both lipid-soluble and water-soluble drugs. Advantages of liposomes extend to reducing toxicities of encapsulated drugs (Bally *et al.,* 1994, Lim, *et al.,* 1997), targeting to specific tissue sites (Loughrey *et al.,* 1993a and b) and enhancing drug efficacy or potency (Mayer, *et al.,* 1993 and 1995).

2.1 LIPOSOME MORPHOLOGY

Liposomes are artificial phospholipid bilayers exhibiting amphiphilic properties. Other molecules, such as cholesterol or fatty acids may be included in the bilayer construction. In complex liposome morphologies, concentric spheres of lipid bilayers are usually separated by aqueous regions that are sequestered from the surrounding solution. The phospholipid component consists of hydrophobic lipid "tails" connected to a "head" constructed of various glycerylphosphate derivatives. The hydrophobic interaction between the fatty acid tails is the primary driving force for creating liposomal bilayers in aqueous solution. The morphology of a liposome may be classified according to the compartmentalization of aqueous regions between bilayer shells (Fig 1.4, p 28). If the aqueous regions are sequestered by only one bilayer, the liposomes are called unilamellar vesicles (ULV). If there is more than one bilayer, the liposomes are termed multilamellar vesicles (MLV). ULV forms are further classified according to their relative size. Thus, there can be small unilamellar vesicles (SUV; usually less than 100 nm in diameter) and large unilamellar vesicles (LUV; usually greater than 100 nm in diameter).

2.2 LIPOSOMES AS DRUG CARRIER

Liposomal drug-delivery systems have shown an important role in oncology during recent years. Several liposomal drugs have been tested in clinical trials or have already been licensed for use. Both preclinical and clinical studies have demonstrated that liposome-encapsulated drugs could reduce toxicities and gain therapeutic efficacy compared to their non-liposomal counterparts. Liposomal encapsulation leads to changes in drug phamacokinetics and biodistribution *in vivo*. In addition, liposomal encapsulation can significantly alter drug-target interactions at a cellular level. Although relatively few liposome anti-cancer drugs have been evaluated in the clinical arena, studies of these new agents are set to expand significantly in the near future (Allen, 1997).

2.2.1 Factors Important for Drug delivery using Liposomes

Several factors such as size, lamellarity and composition of liposomes are crucial for drug delivery using liposomes (Senior, 1987). Generally, large liposomes (more than 100 nm in diameter) have difficulty passing through the walls of normal, mature blood vessels. However, these liposomes escape relatively easy in areas of new vessel formation, such as tumor or inflammation. Therefore, liposomes (50-200 nm) have the potential to deliver their encapsulated drug selectively to tumors, taking advantage of leaky tumor capillaries (Gabizon *et al*., 1990; Vaage *et al*., 1994). Enhanced tumor localization of cytotoxic drug using these liposomes has been shown in animal models and in humans (Gabizon *et al*., 1994; Mori *et al*., 1996).

Different composition of liposomes has led to the production of entities which can serve different clinical roles. Conventional liposomes with rapid uptake by RES system can be used for therapy of viral, bacterial and protozoan infection (de Marie, 1996; Bakker-Wounderberg *et al*., 1994) as well as for the development of vaccines (Alving, 1995). The long circulation of liposomes with preferential extravasation at sites of leaky capillaries has led to the development of novel effective anti-cancer drugs (Gabizon *et al*., 1994). Including enzyme and cytokine in the formulation of liposomes may be exploited for hormone replacement therapy (Anderson *et al*., 1994), gene and antisense therapy (Cooper, 1996, and Woll and Hart, 1995). Coupling antibody molecules to liposomes may improve specific targeting (Park *et al.*, 1995 and Vingerhoeds *et al.*, 1994).

The stability of the liposome structure in plasma is important in terms of drug delivery. It is not only closely related to size, lamellarity, lipid composition and temperature, but also to their interaction with plasma proteins, particularly high-density lipoproteins (Allen and Chonn, 1987; Lasic, 1991). Conventional liposomes are subject to rapid opsonization and uptake by RES. To reduce this effect, early pioneers have tried to saturate liver and spleenic uptake by first giving empty liposomes (Abra and Hunt, 1981). However, it seems that inclusion of certain lipids into the bilayer could stabilize and effectively prolong their circulation time. Therefore, taking place of unsaturated forms of lipids, saturated phospholipids combined with cholesterol were used to generate rigid bilayers, which resulting in longer periods of circulation (Senior, 1987). Moreover, attachment of hydrophilic polymers such as polyethylene glycol to the liposome surface was also found to reduce the rate of clearance (Woodle and Lasic, 1992; Papahadjopoulos *et al.*, 1991).

2.2.2 Stealth Liposomes

The conventional liposome appears to have a very short circulation time due to fast clearance by RES. This has compromised their application in drug delivery to other tissues (Allen, 1994). The long-circulating liposome formulation, stealth-liposome, containing lipids derivatives of polyethylene glycol (PEG) has been introduced to overcome this problem (Allen *et al.,* 1991; Senior *et al.,* 1991) (Figure 1.5, p 29).

The unique properties of stealth-liposomes have made them suitable for cancer treatment. First, compared to conventional liposomes, stealth-liposomes have longer circulation times that allow them to escape leaky tumor capillaries and accumulate in the solid tumor (Papahadjopoulos *et al.,* 1991 and Gabizon *et al.,* 1994). Second, the enhanced lubrication of stealth-liposome enables them to pass across capillaries more easily to reach the tumor interstitial space. Preclinical studies have tested stealth-liposomes for chemotherapy in a number of tumors including murine C26 colon carcinoma (Huang *et al.*, 1992 and Mayhew *et al.*, 1992), murine J6456 lymphoma (Gabizon *et al.*, 1994), Kaposi's sarcoma (Huang *et al.*, 1993), human lung squamous cell carcinoma (Williams *et al.*, 1993) and human prostate carcinoma (Vaage *et al.*, 1994). The results have indicated that the therapeutic efficacy of passively targeted stealth-liposomal formulations of anticancer drugs was greatly improved compared to conventional liposomes.

2.2.3 Immunoliposomes

Attachment of antibody ligands to liposomes can provide a targeting capacity to this drug carrier system. It can modulate its binding to specific antigenic determinants on cells or to molecules in solution. Antibody-bearing liposomes may possess encapsulated components that can be used for detection or therapy (Fig 1.6, p 30). For instance, fluorescent molecules encapsulated within an antibody-targeted vesicle can be used as imaging tools or in flow cytometry (Truneh *et al.*, 1987). Specific antibodies coupled to the vesicle surface can improve diagnostic assays involving agglutination of latex particles (Kung *et al.*, 1985). Liposomes possessing antibodies directed against tumor cell antigens can deliver encapsulated toxins or drugs to the associated cancer cells, effecting toxicity and cell death (Straubinger *et al.*, 1988; Matthay *et al.*, 1984; Heath *et al.*, 1983, 1984). Encapsulation of chemotherapeutic agents within lipid bilayers reduces systemic toxicity and local irritation often caused by anticancer drugs (Gabizon *et al.*, 1986). The liposomal membrane acts as a slow-release agent so that the cytotoxic components do not come into contact with non-tumor cells. Liposomes binding to cells cause internalization and release of the encapsulated drugs. Antibody targeting can increase the likelihood of vesicle binding to the desired tumor cells.

However, there are problems associated with the use of antibody-liposome conjugates for drug delivery *in vivo*. Particularly, since lipid vesicles are huge compare to similar immunotoxin conjugates, their passage to particular tissue destinations may be difficult or impossible. Liposomes are almost entirely sequestered by the reticuloendothelial system. Their ability to pass through tissue barriers to target cells in other parts of the body is limited by their size. If liposomal conjugates can reach their intended destination, their contents are delivered to the cells by endocytosis. Endocytic vesicles arising from the surface of cells, have diameters in the range 100-150 nm. This limits the size of liposomes that can be used to small vesicles that can bind to the cell surface and be internalized efficiently. Large liposomes (>200 nm), by contrast, will not be internalized and therefore will not be able to deliver their contents (Leserman and Machy, 1987).

The promising results on passive targeting of stealth-liposomes encouraged researchers to explore the potential therapeutic applications involving antibody-mediated targeting of liposomes (immunoliposomes). With the aid of antibody ligand, the stealth-immunoliposomes can specifically recognize the tumor cells, locally releasing the encapsulated cytotoxic drugs. Some experiments have demonstrated that with an antibody against lung endothelial cells, stealth-immunoliposomes would localize efficiently in murine lung tissue (Maruyama *et al.*, 1990 and Mori *et al.*, 1995). Moreover, the therapeutic treatment with stealth-immunoliposomes appeared to be effective using doxorubicin entrapped in stealth-immunoliposomes linked to MAb174H.64 to treat murine squamous lung carcinoma (KLN) in mice (Ahmad *et al.*, 1993). Besides the specific targeting, both internalization of immunoliposome-drug complex and 'bystander effect' on the tumor cells lacking the specific epitope might contribute to the mechanism of cytotoxicity (Allen, 1994).

2.2.4 Biotinylated Liposomes

Liposomes containing biotinylated phospholipid components have been used in liposome immunosorbant assay. This is one of the methods that use liposomes with modified surface to form biotin-avidin or biotin-streptavidin complex. (Plant *et al.*, 1989). In this application, the liposome can create a broad surface to facilitate multivalent antigen-antibody interactions, enhancing the total binding efficiency. In addition, it has enormous capacity as a vehicle to carry encapsulated detecting components such as radioisotopes or fluorescent and hence increases the sensitivity of an assay. One small liposome can provide up to 10^5 molecules of fluophore or radiolabels to facilitate enhanced detectability of a binding event. This system, using biotinylated liposomes to detect antigens, can increase the sensitivity of an immunoassay up to 100-fold over the conventional antibody-enzyme ELISA.

Biotinylated liposomes are usually generated by modification of phosphotidyl ethanolamine (PE) components with an amine-reactive biotin derivative, such as NHS-LC-Biotin. The NHS ester reacts with the primary amine of PE residues, forming an amide bond linkage. Since the modification occurs at the hydrophilic end of the phospholipid molecule, after vesicle formation the biotin component protrudes from the liposomal surface. In this configuration, the surface-immobilized biotins are accessible to avidin or anti-biotin antibody present in the outer aqueous solution. Direct biotinylation of intact liposomes could be done, which makes the control of biotin content impossible. Therefore, it is preferred to use biotinylated phospholipid, which allows incorporation of known amounts of biotin into the final liposomal membrane.

After reviewing the literature on these two drug delivery systems, this thesis will describe the research aimed at merging these two systems.

3. DEVELOPMENT AND APPLICATIONS OF BSMAB - RESEARCH GOALS

3.1 OBJECTIVE

Human cancers are often more difficult to treat due to the similarity between the diseased cells and the normal cells. The long-term goal of this research is to develop novel bioconjugates and explore their utility in targeted delivery to the diseased site for diagnosis and therapy.

3.2 HYPOTHESIS

Based on the review of the literature, it was envisaged that bsMAbs could be developed as efficient immunoconjugates for diagnostic and therapeutic applications. Some current applications use avidin/streptavidin and biotin probes. Incorporation of an anti-biotin arm or paratope as one-half of the antibody in the bsMAb construct was to be the common theme in the hypothesis and experimental approach as alternatives to the avidin-biotin strategy. In order to accomplish these goals, a few methodological milestones needed to be accomplished. It was necessary to show that a high affinity monospecific MAb could be generated against biotin which could provide the platform to develop bsMAbs bearing anti-biotin binding arm. It was hypothesized that: a) a bsMAb incorporating enzyme and biotin binding paratope could be developed as a universal immunoprobe for all biotinylated macromolecules with properties similar to or better than avidin or streptavidin, and b) a bsMAb incorporating anti-tumor and anti-biotin paratopes can efficiently pretarget tumor sites. This preloading of the tumor would allow subsequent efficient targeting of $^{99m}TcO_4$ liposomes with biotin incorporated on its surface.

3.3 SPECIFIC AIMS

1. To prepare and characterize a hybridoma that secrets MAb against biotin by conventional hybridoma technology.
2. To develop a simple and efficient micro-electrofusion method to generate quadromas without laborious drug selection procedures.
3. To develop a bsMAb (anti-biotin x anti-HRPO) and use it as an immunoprobe to detect biotinylated macromolecules in different bioassay systems.

4. To prepare ^{99m}Tc-DISIDA loaded liposome and assess its biodistribution in normal Balb/c mice.
5. To establish a tumor-specific drug delivery system combining bsMAb (anti-biotin x anti-squamous carcinoma) and biotin pegylated liposome in a murine KLN 205 model, and also evaluate the role of streptavidin as a clearing reagent.

The next chapter will describe the development and characterization of a hybridoma secreting MAb against biotin. Chapter 3 will focus on the method to generate quadromas by micro-electrofusion without drug selection. Chapter 4 will show a wide variety of applications of one bsMAb bearing anti-biotin and anti-HRPO binding arms. Chapter 5, a novel method is described to load the preformed liposome with model drug ^{99m}Tc-DISIDA (Diisopropyl iminodiacetic acid). Finally, the last chapter will describe the development of a tumor-specific drug delivery system using a bsMAb and biotin pegylated liposome.

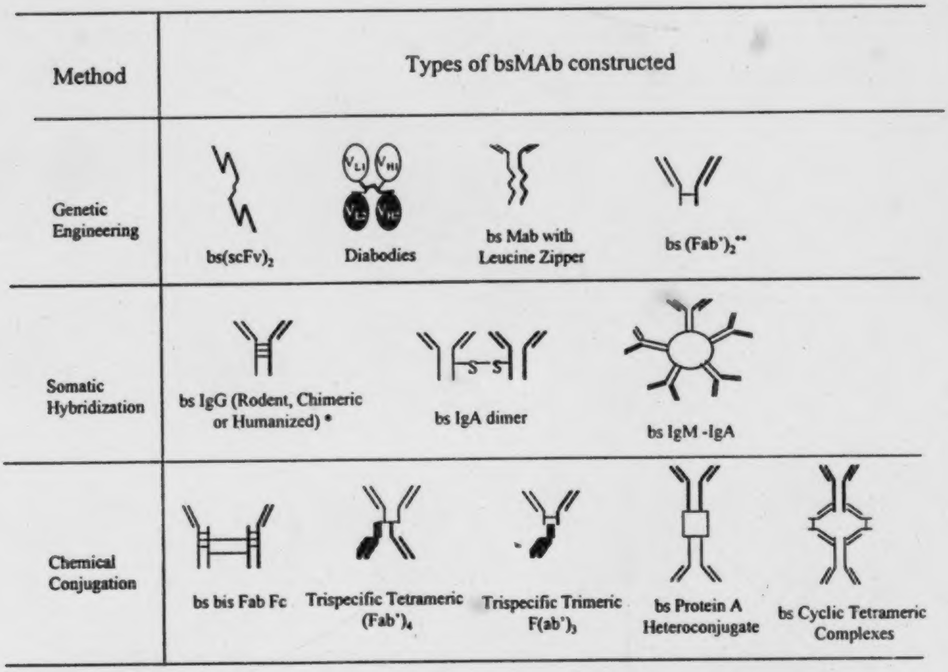

Method
Types of bsMAb constructed
Genetic Engineering
V_{L1}
V_{H1}
V_{L2}
V_{H2}
bs(scFv)$_2$
Diabodies
bs Mab with Leucine Zipper
bs (Fab')$_2$**
Somatic Hybridization
bs IgG (Rodent, Chimeric or Humanized) *
S–S
bs IgA dimer
bs IgM -IgA
Chemical Conjugation
bs bis Fab Fc
Trispecific Tetrameric (Fab')$_4$
Trispecific Trimeric F(ab')$_3$
bs Protein A Heteroconjugate
bs Cyclic Tetrameric Complexes

Figure 1.1 A schematic representation of a variety of bsMAbs. * This has also been constructed by chemical and genetic methods. ** This has also been constructed by chemical methods.

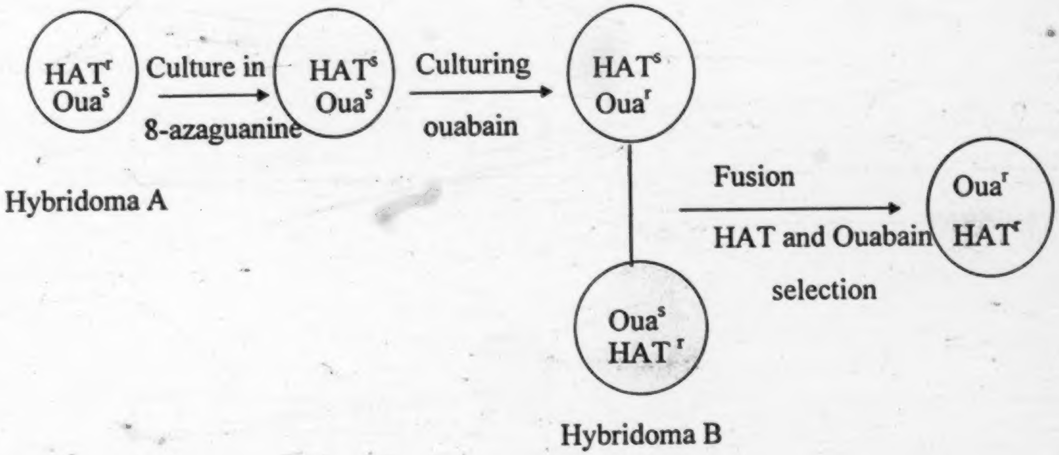

HAT^r
Oua^s
Culture in
8-azaguanine
HAT^s
Oua^s
Culturing
ouabain
HAT^s
Oua^r
Hybridoma A
Fusion
HAT and Ouabain
selection
Oua^r
HAT^r
Oua^s
HAT^r
Hybridoma B

Figure 1.2 Production of quadromas. A HAT sensitive (HAT^S) clone is isolated from one parental hybridoma by culturing in 8-azaguanine. This clone is subsequently made resistant to ouabain (Oua^r) by mutant selection. After fusion with a wild-type hybridoma that produces a different antibody, quadromas are selected by culturing the cells in HAT medium supplemented with ouabain. The unfused parental cells will die due to sensitivity to HAT and lack of resistance to ouabain, respectively.

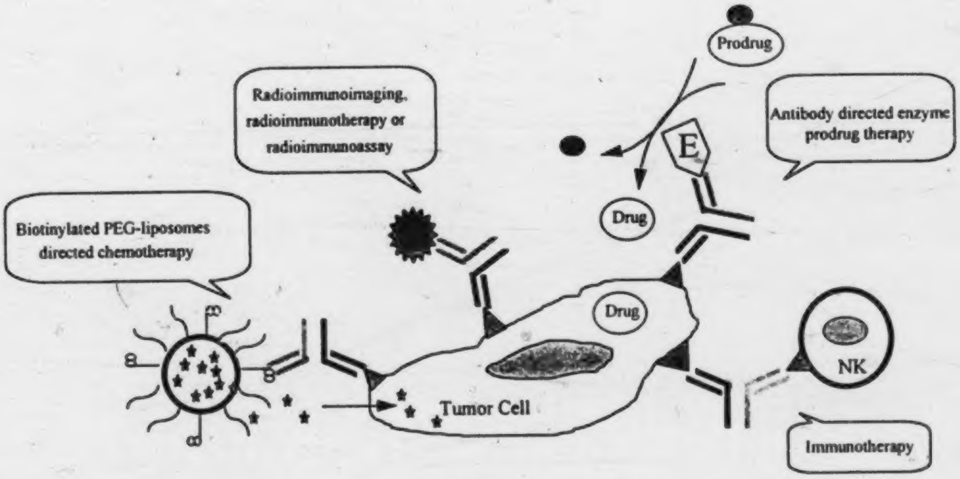

Prodrug
Radioimmunoimaging, radioimmunotherapy or radioimmunoassay
Antibody directed enzyme prodrug therapy
E
Drug
Biotinylated PEG-liposomes directed chemotherapy
Drug
NK
Tumor Cell
Immunotherapy

Figure 1.3 Schematic representation of different applications of bsMAb in therapeutics.

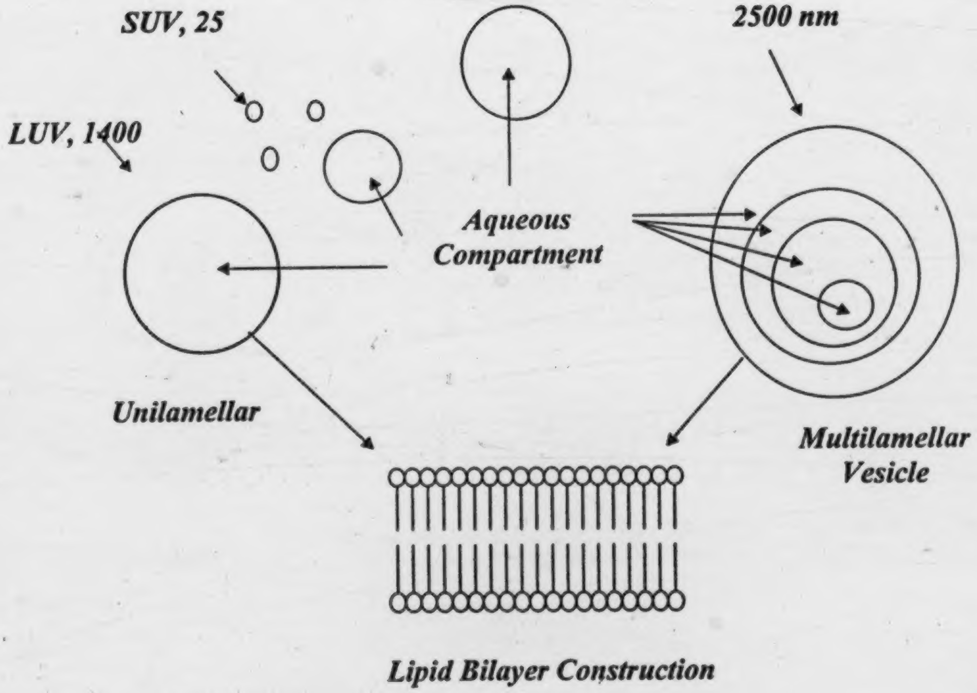

SUV, 25
LUV, 1400
2500 nm
Aqueous Compartment
Unilamellar
Multilamellar Vesicle
Lipid Bilayer Construction

Figure 1.4 Morphology of liposomes and lipid bilayer construction.

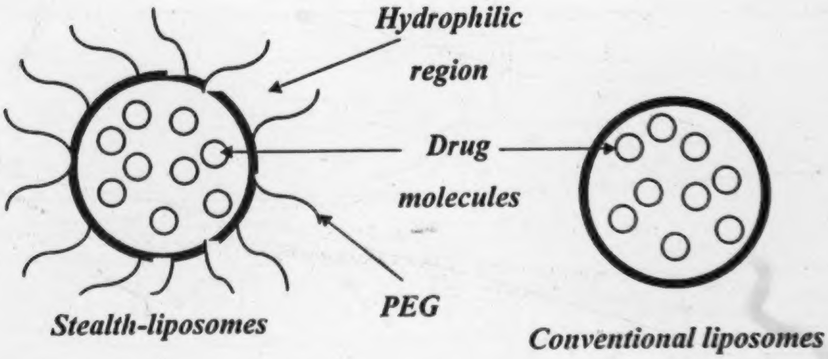

Hydrophilic region
Drug molecules
PEG
Stealth-liposomes
Conventional liposomes

Figure 1.5 Schematic representation of Stealth-liposomes and conventional liposomes. PEG attracts water molecules to the liposome surface and the hydrophilic region prevent the liposome from opsonization by plasma proteins.

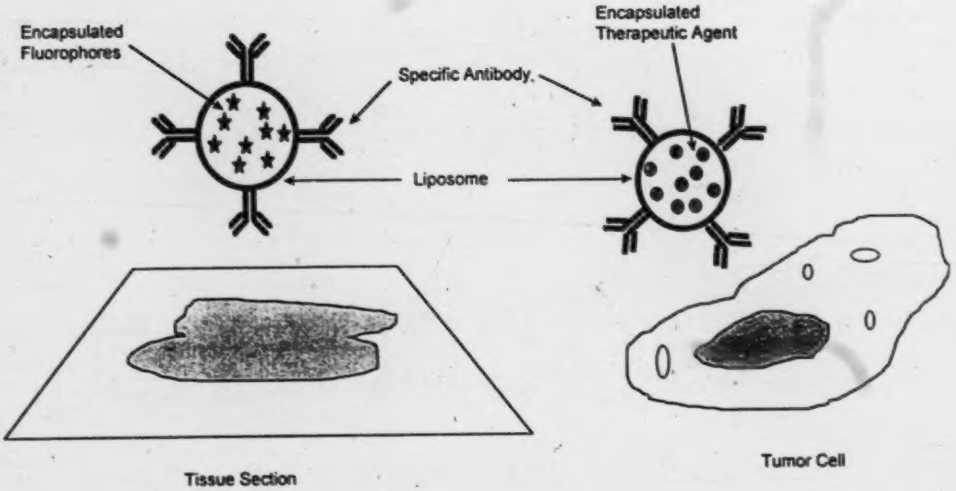

Encapsulated Fluorophores
Specific Antibody
Encapsulated Therapeutic Agent
Liposome
Tissue Section
Tumor Cell

Figure 1.6 Antibody-liposome conjugates may be used as targeting reagents for detection or therapeutic applications. The liposomes may be constructed to contain fluorescent molecules for detection purposes or bioactive agents for therapy. The antibody component targets the complex for binding to specific antigenic.

CHAPTER 2

DEVELOPMENT AND CHARACTERIZATION OF A MONOCLONAL ANTIBODY TO BIOTIN

Part of this chapter has been accepted by Journal of Immunological Methods for publication (1998).

1. INTRODUCTION

D-Biotin (hexahydro-2-oxo-1H-thieno [3,4-d] imidazole-4-pentanoic acid) is a vitamin H (Fig 2.1, p 43), a naturally occurring growth factor present in small amounts within every cell. It is an important component in numerous processes involving carboxylation reactions, wherein it functions as a cofactor and transporter of CO_2. Since biotins and biotin derivatives are relatively small, macromolecules such as protein, DNA and lipids can be modified with these reagents without significantly affecting their physical or chemical properties (Della-Penna *et al.*, 1986).

The biotin/avidin or streptavidin system has one of the highest affinities constants (10^{-15} M) among receptor-ligand interactions (Green, 1963). The strong interaction between biotin and avidin or streptavidin has been applied in many immunological systems for the isolation, localization and visualization of various antigens in addition to drug delivery, lymphocyte stimulation, immunoassays and nucleic acid hybridization assays (for review see Diamandis and Christopoulos, 1991). However, the relatively high background and low specificities restrict the full utilization of this system (Riley and Caffrey, 1990; Wilchek and Bayer, 1988; Diamandis and Christopoulos, 1991).

In this chapter, the development of a mouse hybridoma cell line secreting MAb against biotin will be described. It was used to generate a bsMAb (anti-biotin x anti-

HRPO) which could provide an alternative probe to detect biotinylated macromolecules with lower background noise compared to avidin and streptavidin. Also, it will be used to develop an efficient drug delivery system to solid tumor.

2. METHODS AND MATERIALS

Chemical reagents used for preparation of antigens including d-biotin, keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), ovalbumin (OVA), NHS-LC-biotin [N-hydroxysuccinimidyl-6-(biotinamido) hexanoate], carbodiimide, aqueous pyridine, and 4-hydroxy-azobenzene-2'-carboxylic acid (HABA) were purchased from Sigma Chemical Company, Mississauga, ON, Canada. RPMI-1640 media, L-glutamine, penicillin, streptomycin and fetal bovine serum (FBS) were also from Sigma. Growth factor was from IGEN Inc, Rockville, M.D. USA.

2.1. Antigen Preparation

Biotin was attached to KLH according to the procedure of Berger (Berger, 1979). Biotin 10 mg was suspended in 1.5 mL of 50% aqueous pyridine with constant stirring at room temperature. Carbodiimide 1 mg was dissolved in 5 mL of 50% aqueous pyridine and this mixture was added dropwise to the biotin suspension. Stirring was continued for an additional 30 min at room temperature during which time a clear solution was formed. 5 mg KLH dissolved in 1.5 mL of water was added dropwise to the biotin/carbodiimide reaction mixture and stirring was continued at room temperature for 4.5 h. The mixture was dialyzed for 24 h in 4 L of saline. The prepared biotin-KLH was stored in aliquots and used as an antigen for immunization.

Biotin was covalently attached to BSA using activated biotin. NHS-LC-biotin 10 mg with caproic acid bridge dissolved in 2 mL of DMSO was added into 31 mg of BSA in 18 mL of 0.2 M borate buffer (pH 9.4). Mixture was stirred overnight at room temperature and dialyzed against 4 L of PBS (pH 7.4). Biotin-OVA was prepared by the same method described above. NHS-LC-biotin 10 mg dissolved in 2 mL of DMSO was added into 20 mg OVA in 18 mL of 0.2 M bicarbonate buffer (pH 9.0). Mixture was stirred overnight at room temperature and dialyzed against PBS (pH 7.4). Biotin-

BSA and biotin-OVA prepared here will be used as solid phase capture reagent for anti-biotin antibodies in the detective immunoassays.

The degree of biotinylation was estimated by the spectrophotometric method using HABA assay (Green, 1970). Briefly, the absorbance at 500 nm of 1 mL solution of avidin-HABA complex (0.25 mM HABA in 0.1M phosphate buffer containing 0.2 mg/mL avidin) was measured before and after the addition of 50 μL of biotin-protein conjugate. The difference in absorbance is as a function of the biotin concentration. The concentration of biotin in the sample was calculated from biotin standard curve.

2.2. Intrasplenic Immunization

The protocol of this short immunization method is shown in Table 2.1. The 8-week old male BALB/c mice were immunized by two intraperitoneal injections, one week apart, of 50 μg of biotin-KLH emulsified in Freund's complete adjuvant. Freund's incomplete adjuvant was used in the second injection. Three days after the second injection, the mice were injected intraperitoneally with 100 μg of the same antigen in Freund's incomplete adjuvant. The serum titer of the anti-biotin antibodies were monitored by direct ELISA as described below to choose the best mouse. Three days prior to hybridoma cell fusion, the mice were boosted by surgically delivering 50 μg of biotin-KLH to the spleen (Spitz, 1984). After 3 days, spleenocytes were obtained for the myeloma cell fusion. Mouse serum was prepared at the same time to provide a positive control in a subsequent immunoassay.

2.3 Hybridoma Fusion

The hybridomas were generated by fusing the spleen cells taken from an immunized mouse with SP2/0 myeloma cells. SP2/0 is a myeloma cell line which does not secrete immunoglobulin and is deficient in hypoxanthine guanine phosphoribosyl transferase (HGPRT). The conventional cell fusion protocol by using polyethylene glycol (PEG) as a fusion reagent is described as follows (Kohler and Milstein, 1975):

Approximately 1x10^7 SP2/0 myeloma cells were mixed with spleen cells at a 1:5 ratio and washed three times with RPMI 1640 without FBS. Following the last wash, the cell mixture was centrifuged at 500 x *g* for 5 min at room temperature and the supernatant was discarded completely. Prewarmed PEG (MW 1700) 1 mL was added dropwisely to the cell pellet for 90 sec. Then the PEG solution was diluted slowly by adding 1 mL of RPMI 1640 medium for 1 min followed by another 3 mL for 1 min and 10 mL again with gentle shaking. After 5 min, the fusion was stopped by adding 10 mL RPMI 1640 with 20% FBS. The cells were centrifuged and washed twice with RPMI 1640 with 20% FBS. Finally the pellet was resuspended in HAT selection media. The selection media contain RPMI 1640 supplemented with 2 mM L-glutamin, 50 U/mL penicillin and streptomycin, 20% v/v of FBS, HAT (0.1 mM sodium hypoxanthine, 0.4 μM aminopterin and 16 μM thimidine) 10% v/v growth factor (ORIGEN), OPI (oxalacetic acid, sodium pyruvate and bovine insulin). The cells were plated into 96-well tissue culture plate at concentration of 1.5 x 10^5 cells/well and incubate in a humidified, 5% CO_2 incubator at 37°C.

2.4 Screening by Direct ELISA

In order to screen hybridomas which secrete anti-biotin antibodies, direct ELISA methodology was used. Standard 96-well ELISA plates (Nunc, Grand Island, NY) were coated with 1 μg/well biotin-BSA overnight at room temperature as a solid-phase capture reagent. The nonspecific binding sites were blocked by 200 μL of 3% BSA/PBS overnight at 4 ^{0}C. The wells were washed with PBS/Tween-20 (0.02%) and 50 μL of the hybridoma cell supernatant was added and incubated at room temperature for 1 h. This was followed by washing 5 times with PBS/Tween-20. Goat anti-mouse second antibody Ig(G+A+M) conjugated with horseradish peroxidase (HRPO) 100 μL was added and the plates were incubated for 1 h at room temperature. After another 5 washings, 100 μL of ABTS + H_2O_2 (Pierce, USA) was added and plates were read after 30 min in a microplate reader (Molecular Devices Corporation, Sunnyvale, CA). The optical density was measured at 405 nm.

2.5 Isotyping of MAbs

1) ELISA method. The isotype of anti-biotin antibodies were determined using direct ELISA with different anti-isotype secondary antibodies. Standard 96-well plates were coated with 1 µg/well of biotin-BSA as solid phase antigen at room temperature overnight. The nonspecific binding sites were blocked with 3% BSA/PBS. The wells were washed 5 times with PBS/Tween-20 (0.02%) and 100 µL of anti-biotin antibodies from three clones was added and incubated at room temperature for 1 h. After washing, 100 µL of different goat anti-mouse isotype antibodies [including anti-IgM, anti-IgA, anti-IgG1, anti-IgG2a, anti-IgG2b, anti-IgG3 and anti-Ig(G+M+A)] (Sigma) conjugated with HRPO at 1:1000 dilution was added and incubated for 1 h at room temperature. Following a final wash, color was developed by adding ABTS + H_2O_2 for 30 min.

2) Isotyping Kit. The supernatants from three clones were diluted 1:100. 150 µL of the diluted sample was added into each development tube and incubated at room temperature for 30 sec. The tube was agitated briefly so that the colored latex was completely resuspended. The strip was added into development tube for 5 min.

2.6 Determination of Relative Binding Efficiency

The relative binding efficiency of P54 clones was determined by a residual titration immunoassay. ELISA microtiter plates were coated with 1 µg/well biotinylated BSA as the solid phase capture reagent at room temperature overnight. The plates were blocked by 3% BSA/PBS. 50 µL varying concentrations of biotinylated BSA and 50 µL supernatant containing 0.5-1 µg of antibody from three clones were mixed and incubated for 4 h at room temperature. Then the mixture was added into the biotinylated BSA pre-coated ELISA plates to detect residual unbound MAb by ELISA. The relative affinity was determined by the concentration of free biotinylated BSA, at which a 50% reduction of total binding was achieved.

2.7 Cloning of Hybridoma

The best positive clone was selected based on the relative affinity of the anti-biotin antibody and high stability in the cell culture. The selected clone was recloned twice by limiting dilution to ensure monoclonality. The limiting dilution was done as follows: The hybridomas were resuspended in a standard RPMI-1640 media. After counting the cells, a serial dilution was made to achieve a final concentration of 3 cells/mL. Then 100 μL of cell suspension and 100 μL of RPMI-1640 media with 10% growth factor and 10% FBS were added to each well in a 96-well plate. The supernatant of each clone was tested for the anti-biotin antibody activity after 7-10 days, and the cloning efficiency was calculated as follows:

Cloning efficiency % = Number of positive clones x 100 / Number of clones (1)

The limiting dilution was continued until the cloning efficiency reached 100%, and the clone was then expanded and frozen in liquid N_2 for the future use. The same technique was also used for preparing monoclonal quadromas producing desired bsMAbs.

2.8 Ascites Production and Purification

Male BALB/c mice were primed with 0.5 mL of Pristine (2, 6, 10, 14-tetramethylpentadecane) by intraperitoneal injection (Harlow and Lane, 1988). After two weeks, the monoclonal hybridom P54.1R cells 1 x 10^6 in 0.2 mL saline were inoculated peritoneally into BALB/c mice. Ascites fluids were collected in tubes containing heparin two weeks after inoculation. The MAbs from the ascites fluids were diluted by PBS and purified by ammonium precipitation and ion exchange column (Suresh, 1986a).

Briefly, solid ammonium sulfate was gradually added with stirring to achieve 50% salt saturation and the solution was continuously stirred at 4^0C overnight. The solution was centrifuged at 3000 x *g* for 30 min and the pellet was collected. The precipitated immunoglobulins were dissolved in 10 mM sodium phosphate buffer (pH 7.4) and dialyzed against 4 L of the same buffer. The dialyzed sample was loaded on to a DE52 column equilibrated with 10 mM sodium phosphate buffer (pH 7.4). The column was washed to remove the unbound protein until no protein can be detected in the eluent. The immunoglobulins were eluted using linear ionic gradient from 10 mM to 100 mM sodium phosphate buffer. The protein concentration was measured by UV detector at 280 nm. The antibody activity was determined by direct ELISA as described above. The fractions with highest antibody activity were pooled and the purity was analyzed by reduced SDS-PAGE (Laemmli, 1970).

2.9 Determination of Dissociation Constant

ELISA was used to determine the dissociation constant (Kd) of antibody and antigen (Friguet, *et al.*, 1985). The antigen at various concentrations (8×10^{-9} M to 2×10^{-7} M) was first incubated in solution with the antibody at constant concentration (6×10^{-9} M). After 2 h incubation at room temperature and overnight incubation at 4^0C, 100 μL of each mixture was transferred into microtiter plate pre-coated with 1 μg/well biotinylated BSA and incubated for 1 h at room temperature. After washing with PBS plus 0.02% Tween 20, the bound antibody was detected by adding goat anti-mouse antibody conjugated with HRPO. The Kd was determined from the slope of the Scatchard plot.

3. RESULTS AND DISCUSSION

3.1 Preparation of Biotin-Carrier Conjugates

Biotin has a very high affinity with avidin and streptavidin ($K_a = 1.3 \times 10^{-15}$ M). The binding occurs between the bicyclic ring of biotin and a pocket within each of the four subunits of avidin. The valeric acid portion is not involved or required for the interaction (Green, 1965; Wilchek and Bayer, 1988). This characteristic allows modification of the valeric acid side chain without affecting the binding potential toward avidin and streptavidin. D-biotin is the basic building block for constructing biotinylation reagents. The molecule may be attached directly to a protein via its valeric acid side chain, or derivatized at this carboxylate with other organic components to create spacer arms and various reactive groups.

Biotin is a small molecule, although normally able to interact with the products of an immune response, often cannot cause an immune response on their own. They are actually incomplete antigens called haptens. But after coupling them to a suitable carrier molecule, they can be made immunogenic to elicit antibody production. Carriers typically are antigens of higher molecular weight that are able to cause an immunological response when administered *in vivo*. The most common carrier proteins in use today are KLH (MW 4.5 x 10^2 to 1.3 x 10^4 kD) , BSA (MW 67 kD), OVA (MW 43 kD) and thyroglobulin (MW 660 kD). Since an antibody response would be directed against both the carrier and the attached hapten, an ELISA done to measure the antibody response specifically to hapten must not utilize the same carrier in the conjugate coated on the microplates. If the carrier conjugate used for the ELISA is identical to that used in the original immunization, the rest results will be skewed by the contribution of carrier-specific antibodies. For this reason, a non-relevant carrier which is not recognized by the products of the immune response must be coupled with hapten and used for ELISA test. In this experiment, KLH was chosen as a carrier to immunize mice to develop specific antibody to biotin, BSA and OVA, however, were used as non-relevant carriers in ELISA tests designed to quantify the antibody response.

3.2 Determination of Degree of Biotinylation

Determination of the degree of biotinylation is often important to optimize a particular biotin-anti-biotin or biotin-avidin assay system and ensure reproducibility in the biotinylation process. The most commonly used method for measuring the extent of biotin modification makes the use of 4'-hydroxyazobenzen-2-carboxylic acid (HABA) assay (Green, 1965). In the absence of biotin, the dye is able to form specifically noncovalent complexes with avidin at its biotin binding sites. After binding to avidin in aqueous phase, avidin/HABA complex exhibits a characteristic absorption band at 500 nm. The addition of biotin to this complex results in displacement of HABA from the binding site, since the affinity constant of the avidin/biotin interaction (10^{15} M^{-1}) is much higher than that for avidin/HABA (6×10^{6} M^{-1}). As HABA is displaced, the absorbance of the complex decreases proportionally. Thus, the amount of biotin present in the solution can be determined by plotting the avidin/HABA absorbance at 500 nm versus the absorbance modulation with increasing concentrations of added biotin. Comparing an unknown biotin-containing sample to this standard response curve can result in the determination of the biotin concentration in the sample.

The molar ratio of biotin to OVA and BSA carrier were 3.5 and 4.0 respectively according to this method. However, the molar ratio of biotin to KLH was not determined because KLH is not a single component.

3.3 Development of Hybridoma P54.1R

MAbs to biotin have been prepared using biotinylated KLH as the immunogen (Fig 2.2, p 44). The intrasplenic immunization was demonstrated to be a rapid and effective method to produce MAbs. A total of 3 mice was immunized with biotinylated KLH according to the protocol described in Table 2.1, p 47.

The mouse sera were tested for anti-biotin antibody activity by direct ELISA. The mouse with the highest antibody titer was selected for hybridoma fusion, since the high titer of anti-biotin MAb might indicate that more B cells producing anti-biotin antibodies or higher affinity of anti-biotin MAb were probably present in the immunized mouse. The splenocytes collected from this immunized mouse were then fused with SP2/0 myelomas by PEG. After the fusion, cells were seeded in microtiter plates and selected in HAT media, wherein only the hybridomas can survive. 2 weeks after fusion, the visible clones were screened for the hybridoma producing anti-biotin MAb. The primary screen was performed on biotin-BSA coated ELISA plates. The first incubation was to hold the mouse immunoglobulins from the supernatants of each clone to the plates and then the bound immunoglobulins were detected by goat anti-mouse antibody conjugated with HRPO.

The fusion efficiency of the mouse splenocytes with the mouse myeloma SP2/0 was relatively high. Hybridoma cell growth was observed in 60% of the wells with a total of 384 primary clones appeared in 96-well plates. When tested by ELISA, the presence of hybridoma secreting anti-biotin antibody was detected in 30% of hybridomas. About 20 positive clones with high anti-biotin activity were subsequently expanded into 24-well plates and 6-well plates. The activities were tested continuously during the transition periods since the hybridomas may lose the ability to secrete antibodies. The best three clones (P54.1R, P54.2R and P54.3R) were finally selected and their ELISA results were in Table 2.2, p 48.

They were recloned until the hybridomas continued to produce 100% positive wells at the limiting dilution of less than 0.3 cell/well. All of these three clones were stored in liquid N_2 for the future use. The isotype and binding affinity were determined in order to choose the best one for developing quadromas.

3.4 Isotype of anti-biotin antibody

Early characterization of a MAb's isotype is important for a number of reasons. First, an antibody's isotype can determine the simplest method of purification. For example, IgG2a and IgG2b bind protein A and therefore can be purified by protein A affinity chromatography. However, this method may not be suitable for IgG1 monoclonals because they do not bind protein A well under standard conditions. Second, isotype characterization also reveals an antibody's structure, which may make it undesirable in some applications. For example, IgM's exist as pentamers composed of five 180-kD subunits, therefore these unstable IgM monoclonals are not suitable to make bsMAb and often too large for applications that require monomers like IgG isotypes. Fourth, an antibody's isotype determines the best method of preparing antibody fragments by proteolysis. The isotypes of MAbs secreted by these three clones were all IgG1 (γ, λ) determined by ELISA (Table 2.3, p 49) and commercial isotyping kit.

3.5 Relative Binding Efficiency of Anti-Biotin Antibody

Before a hybridoma is generated for further development of quadromas, it is important to know the binding affinity of the antibody it secretes, because a bsMAb with a high affinity to biotin is required for developing an immunoprobe or a drug delivery system. Instead of measuring absolute antibody affinity which requires purified antibody, the relative affinity of these antibodies could be simply compared using cell culture supernatants collected from each clone. Fig. 2.3 shows the relative affinities of these antibodies from three clones. The relative affinities were determined by a residual titration assay. The antibodies were first incubated with soluble biotinylated BSA. Residual antibodies were measured by binding to solid phase biotinylated BSA in ELISA. With increasing the concentration of soluble biotinylated BSA, fewer residual antibodies would be available to bind the solid phase biotinylated BSA. Since a wide range of soluble antigens were used, the relative higher affinity MAb would be saturated with antigen resulting in negligible residual MAb for subsequent titration. P54.1R was chosen for further studies based on the highest relative affinity to biotinylated BSA among the three clones and its residual antibody fraction falls to zero at concentrations that are 2-3 orders of magnitude lower than for the other two clones.

3.6 Preparation of Anti-Biotin Antibody

To obtain a large amount of P54.1R antibody for further studies, hybridoma P54.1R was inoculated into BALB/c mice and 7 mL of mouse ascites containing anti-biotin antibody was produced. The anti-biotin activity could be detected when the mouse ascites was diluted up to 10,000 times. P54.1R was purified using ammonium sulfate precipitation and ion exchange chromatography (DE-52). The fractions collected from DE-52 column were tested for anti-biotin activity and the fractions with high anti-biotin activity were analyzed by reduced SDS-PAGE. The results indicated that fractions 5, 6 and 7 contained fewer contaminants (data not shown). In all, about 5 mg of anti-biotin antibody was generated and kept for the future studies.

3.7 Dissociation Constant Determined by ELISA Method

The dissociation constant of P54.1R to biotin was determined by ELISA (Friguet, *et al.*, 1985). It has the advantage of using native antibody eliminating the possibility of affinity alteration due to chemical modification, such as iodination, biotinylation or enzyme labeling. After equilibrium binding between MAb and antigen, the residual antibodies were simply quantitated in a conventional ELISA by standard calibration curve. The bound antibody is calculated by subtracting the free from the total antibodies. The solution phase biotinylated BSA had 4 moles of biotin per mole of BSA which is a low derivatization ratio in relation to the 57 free NH_2 groups in the primary sequence of the protein. Hence, it is likely that the low biotin ratio and random spatial distribution result in only one Fab arm of MAb involved in binding. The binding affinity of P54.1R to biotin was equal to the calculated avidity value 6.1×10^{-9} M from the Scatchard plot (Fig 2.4 p 46).

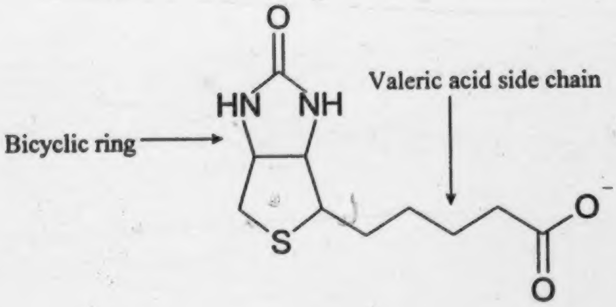

O
HN
NH
Valeric acid side chain
Bicyclic ring
S
O
O

Figure 2.1 Structure of D-Biotin (MW 244.31 D).

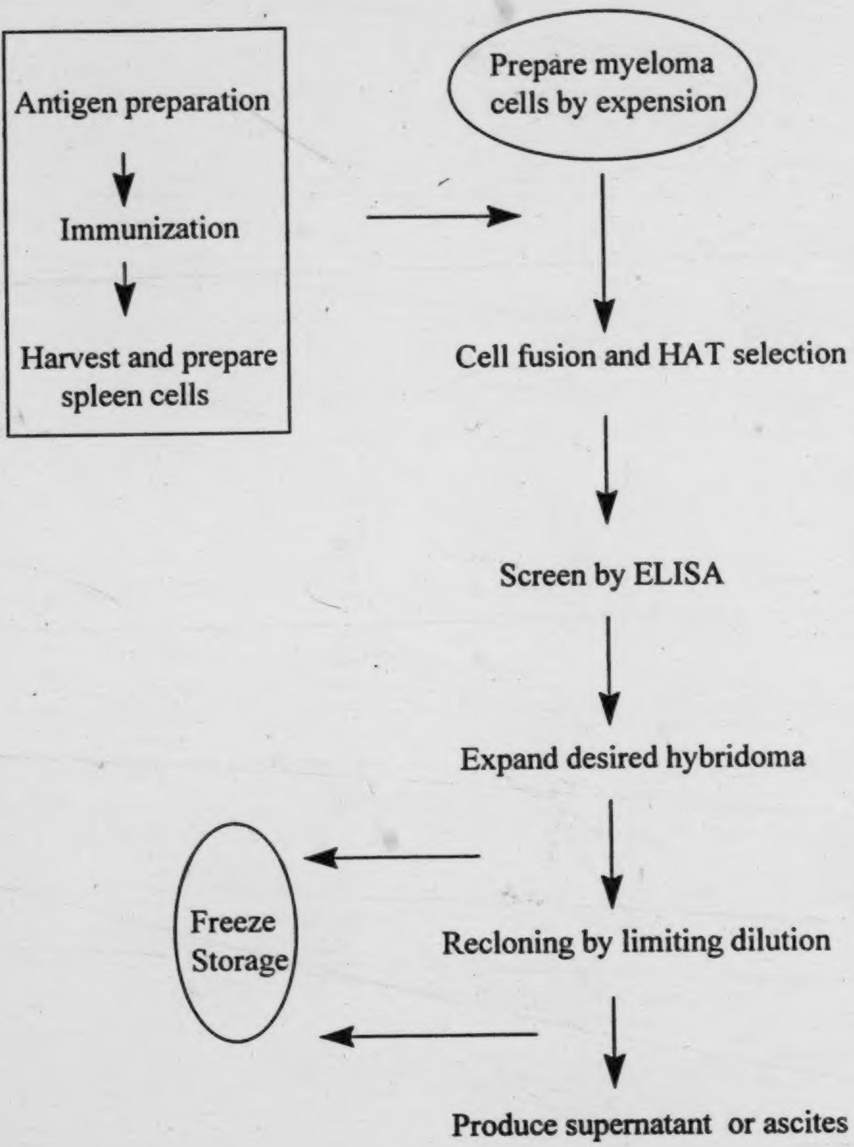

Antigen preparation
Immunization
Harvest and prepare spleen cells
Prepare myeloma cells by expension
Cell fusion and HAT selection
Screen by ELISA
Expand desired hybridoma
Recloning by limiting dilution
Produce supernatant or ascites
Freeze Storage

Figure 2.2 Schematic representation of conventional hybridoma technology.

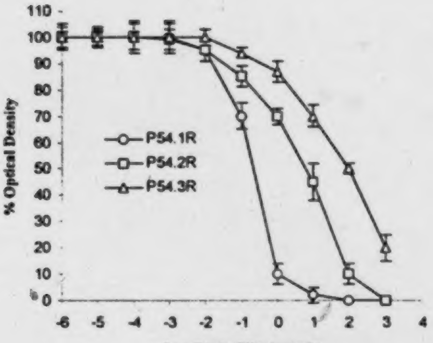


Figure 2.3 Relative affinities of anti-biotin antibodies for biotinylated BSA were determined by a residual titration immunoassy (n=4). The monoclonal antibodies were incubated with biotinylated BSA at varying concentrations for 4 h and subsequently the residual unbound MAb was reacted with the same antigen on the solid phase. Percentage of optical density represents the ratio of the signal generated from ELISA plate in the presence to the absence of competing solution phase antigen.

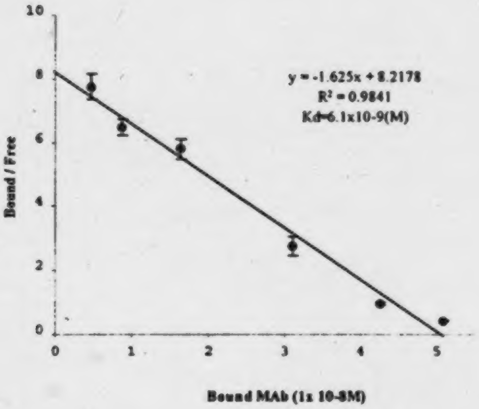


Figure 2.4 Scatchard plot of monospecific MAb P54.1R against biotin (n=4). Various concentrations of MAb and biotinylated OVA were mixed and incubated overnight to establish equilibrium and aliquots were transferred to biotinylated OVA coated plate to estimate unbound MAb in direct ELISA assay.

Table 2.1 Immunization Protocol for Generation of MAb against Biotin

Time (day)	Amount of Antigen[a] (μg)	Adjuvant	Volume (mL)	Route
1	50	FCA[b]	0.2	IP[e]
7	50	FICA[c]	0.2	IP
10	100	PBS[d]	0.5	IP
11[g]	Serum Test			
12	50	PBS	0.15	IS[f]
15	Cell Fusion			

[a] Biotin-KLH

[b] Freund's complete adjuvant (FCA) containing mycobacteria may modulate releasing rate of the antigen and thus improve immunization efficiency.

[c] Freund's incomplete adjuvant (FICA) dose not contain mycobacteria and may also improve immunization efficiency.

[d] Phosphate buffered saline

[e] Intraperitoneal (IP) injection

[f] Intrasplenic (IS) injection

[g] Serum anti-biotin antibody titer determined by ELISA

Table 2.2 Screening of Primary Hybridomas for Anti-biotin Reactivity by ELISA

P54.1	P54.2	P54.3	Positive[b]	Negative[c]
1.101[a]	1.001	0.911	0.952	0.065
1.211	0.982	0.854	0.992	0.076
1.103	1.046	0.871	0.945	0.079

a Optical absorbance measured at 405 nm.

b Serum obtained from antigen immunized mouse was used as positive control.

c Normal mouse serum used as negative control.

Table 2.3 Isotype of Anti-biotin Antibodies from P54 Clones.

	IgM	IgA	IgG1	IgG2a	IgG2b	IgG3	IgG+M+A
P54.1	–[c]	–	+[d]	–	–	–	+
P54.2	–	–	+	–	–	–	+
P54.3	–	–	+	–	–	–	+

a “–” optical obsorbance at 405 nm lower than 0.1.

b “+” optical obsorbance at 405 nm higher than 0.5.

CHAPTER 3

A RAPID NON-SELECTIVE METHOD TO GENERATE QUADROMAS BY MICROELECTROFUSION

This chapter has been published in Journal of Immunological Methods, 187, 1-7, 1995.

1. INTRODUCTION

Hybridoma technology established the route to produce continuous cultures secreting MAbs (Kohler and Milstein 1975). Since this milestone accomplishment, several new second generation MAbs have been made such as bsMAbs, chimeric MAbs, CDR grafted MAbs, bifunctional MAbs (fusion proteins), abzymes, single chain Fv and domain antibodies (dAbs).

BsMAbs with two distinct binding specificities have shown great promise for immunoassays (Suresh, *et al.*, 1986), immunohistochemistry (Milstein and Cuello, 1983), cell targeting (Lanzavecchia and Scheidegger, 1987), complement-mediated cytotoxicity (Wong and Colvin, 1987), targeting of toxins or drugs to cells (Raso and Griffin, 1981) and molecular hybridization (Auriol *et al.*, 1994). BsMAbs are produced mainly by three methods: chemically linking two antibody molecules, fusion of two different hybridoma cell lines or recombinant DNA based approaches. Quadromas produced by cell fusion can provide continuous cultures secreting bsMAbs and has its advantages over other methods. However, conventional cell fusion requires parental cells to be sensitive to a selection medium, which is a time consuming process.

This chapter will describe a rapid and non-selective method to generate quadromas from two established hybridoma cell lines by micro-electrofusion. This protocol has reduced the time required to make quadromas from 6-12 months to a couple of weeks, and hence could make bsMAbs widely available as routine reagents.

2. MATERIALS AND METHODS

2.1 Hybridoma Cell lines

In this study the following hybridoma cell lines producing antibodies against (i) horseradish peroxidase:YC6/41 (Milstein and Cuello, 1983); (ii) biotin: P54.1R, developed in our laboratory; (iii) head and neck squamous carcinoma:174H.64 (Samuel *et al.*, 1989); and (iv) adenocarcinoma:170H.82 (Longenecker *et al.*, 1987) were used. The anti-biotin and the tumor reactive MAbs are mouse IgG1 monoclonal antibodies, while the anti-peroxidase cell line secretes a rat IgG2a MAb.

2.2 Microelectrofusion

The two desired hybridoma cell cultures were combined at a 1:1 ratio and the mixture was centrifuged at 200x g for 8 min to obtain a cell pellet. The supernatant was removed and the cells were washed twice with fusion media (0.3 M glucose, 0.1 mM Ca^{2+} and 0.1 mM Mg^{2+}). The cells were resuspended in fusion media to a final concentration of $1x10^5$ cells/mL. A meander slide was sterilized using 95% ethanol and placed on an inverted microscope stage housed in a class 100 (Type IIA) biohazard hood. The micrograbber cables were attached to the terminus on the slide and to an Electroporator model ECM 200 (BTX Inc., San Diego, CA). An appropriate amount of the parental hybridoma fusion mixture was pipetted on to this slide.

Electrofusion was carried out under following electrofusion settings as recommended in the manual. Alignment amplitude AC 8.0 V; time 7 sec; field strength 400 V/cm; electrofusion amplitude DC 50 V; pulse width 30 μsec; number of pulses 2. Following electrofusion the cells on the chamber were aspirated with a sterile microtip and pipette, and transferred to a sterile petri dish with 20 mL RPMI-1640 medium (GIBCO-BRL, N.Y.) containing 20% FBS, 1% penicillin-streptomycin (GIBCO-BRL, N.Y.) and 10% Origen growth factor (IGEN, Inc., M.D.). After 30 min incubation (5% $C0_2$ at 37 ^{0}C), the cells were seeded at 200 μL/well into a 96 well plate and incubated. Quadroma clones appeared in 10 to 14 days and the supernatants were tested for bsMAbs production by an ELISA method (Suresh *et al.*, 1986b).

2.3 Bispecific MAb Enzyme Immunoassays

In the bsMAb sandwich screening assay, standard 96 well ELISA plates (Nunc) were coated with 100 μL of a 1:1000 dilution anti-170 or anti-174 idiotype antibody per well and incubated at room temperature overnight. The wells were washed 5 times with PBS/Tween-20 (0.02%). The wells were blocked with 200 μL of 3% BSA in PBS (pH 7.4) overnight at 4 ^{0}C. Quadroma cell supernatant 50 μL (1:100 dilution) mixed with 50 μL HRPO (10 μg/mL) was added and incubated at room temperature for 1 h. This was followed by washing 5 times with PBS/Tween-20. 2, 2'-azino-di [3-ethylbenzthiazolin sulfonate] (ABTS) + H_2O_2 100 μL (Pierce, USA) was added, and plates were read after 30 min in a microplate reader. This basic bsMAb assay was changed according to the type of quadroma cell supernatant used (Table 3.1, p 60). In order to detect bsMAbs bearing an anti-biotin arm, the direct assay was performed as above except the standard 96 well ELISA plates were coated with 1 μg biotinylated BSA as the solid-phase capture reagent.

3. RESULTS AND DISCUSSION

Quadromas were generated by microelectrofusion procedures using the BTX meander slide (Fig 3.1, p 59). This plastic microslide has an electrode grid coated on its surface with the anodes and cathodes separated by 0.2 mm. The stems of the electrode can be connected to the ECM 200 electrofusion apparatus by two micrograbber cables. The slide was sterilized with ethanol, air dried and placed on the inverted microscope housed in a biohazard hood. This allowed for observation of the formation of pairs or a string of cells between the electrodes due to AC voltage applied. Fusion of cells in pairs or more can be observed upon introducing the DC pulse resulting in coalescence of the cytoplasm. Unfused small cells and the fused large cells can both be seen soon after electrofusion. Finally, three sets of quadromas were developed. One set was made by fusing a hybridoma secreting (i) anti-adenocarcinoma antibodies (170H.82) or (ii) anti-head and neck squamous carcinoma (174H.64) with a hybridoma secreting anti-HRPO antibodies (YP4), and the other with a hybridoma secreting anti-biotin antibodies. The third type of quadroma specifying both anti-HRPO and anti-biotin binding arms was also generated (Table 3.1). Five different quadromas were thus generated in less than a month and were identified by enzyme immunoassay according to the different types of bsMAb assays specified in Table 3.1.

The results of the bsMAb ELISA are shown in Table 3.2, p 61. As described in the materials and methods section, this is a bridge immunoassay that takes advantage of the dissimilar binding properties of the two Fab arms of a bsMAb. This assay, while being specific for hybrid immunoglobulins, suffers from potential reduced sensitivity in the presence of excess monospecific immunoglobulins which are also secreted by the putative quadromas. The problem is further magnified due to the total absence of drug selection procedures in this method. Thus, unfused hybridoma cells in the primary clones can add to the parental monospecific MAb secreted by quadromas and hence compete for binding to the solid phase antigen in the bsMAb bridge ELISA. If the solid phase antigen is limiting, this competition and saturation by monospecific MAb (sometimes with higher avidity) can obscure the detection of bsMAb secreted by quadromas in primary clones. Therefore, it is advisable to have solid phase antigen in excess and a low volume or dilution of the clonal supernatant to detect the desired bsMAb. Despite these theoretical limitations of low sensitivity, it was possible to detect the presence of quadroma clones and reclone them twice soon after identification to avoid loss from the overgrowth of the unfused parental hybridomas. The optical density values at 405 nm in all the five different bsMAb ELISA were clear strong positives with a background value of 0.07 when the corresponding monospecific MAbs were used.

The real advantage of this procedure is to make 5 quadromas in a matter of only 2-4 weeks. This is certainly dependent on the availability of well established hybridomas and a screening method along with sufficient reagents to detect bsMAbs. Prior to the optimization of this simple non-selection procedure we routinely spent 3-6 months to make quadromas (Suresh *et al.*, 1986a and b; Kreutz and Suresh, 1995).

The quadromas were optimally generated with 500 cells of each fusion partner hybridoma (Table 3.3, p 62). Different amounts of cells in varying volumes were tried with different seeding ratios. When the number of cells seeded were increased, the number of clones generated increased simultaneously. However , the seeding at the highest density of 100 cells/well did not help identify any quadromas, perhaps due to overgrowth of unfused cells. It can be seen in Table 3.3 that at the highest seeding, all of the 96 wells had clones, but we were unable to detect any bsMAb positive clones. While quadroma clones may have been generated, their ELISA detection was probably obscured by monospecific MAb saturation of solid phase antigen.

The yield or frequency of bsMAb secreting clones were about 1- 4 x 10^{-3} based on the total input cells used for fusion. However one should also look at the yield of quadromas based on the total numbers of clones growing in the 96 well, which increases the frequency to 10 - 40% (Table 3.3). Not all the wells show clonal growth even though 5-10 cells were seeded per well. This is probably due to a combination of factors such as (a) the deleterious effect of electrofusion (b) non-ideal cell culture conditions (c) fusion of more than 2 cells which may have growth problems and (d) the low volume of the cell mixture that was used on the slide (10-50 μL) which may cause some cell death due to drying or lower cell recovery after aspiration of the droplet. Despite all these potential problems, we were able to develop and identify quadromas for subsequent recloning in two cycles. The stability of the quadromas was generally good and their recloning efficiency at the second reclone is shown in the final column of Table 3.3.

These quadromas were subsequently grown for 3-6 months and made ascites in mice for bulk preparation of the bsMAbs for further *in vitro* and *in vivo* studies. Seeding at limiting dilutions of 1 cell/well did not consistently show clonal growth, although one would theoretically expect additional advantage in an instant cloning step as part of primary seeding. The quadromas identified were eventually recloned twice and stored in liquid N_2.

Historically, the first bispecific antibodies were generated by chemically coupling two polyclonal antibodies (Nisonoff and Rivers, 1961). Monoclonal bispecific constructs have been made from whole antibodies as well as their fragments using bifunctional crosslinking reagents. Trispecific antibodies have been developed incorporating three different Fab fragment specificities utilizing a chemical crosslinker. Alternatively protein crosslinkers have also been used to make bsMAbs, using protein A or formation of unique cyclic tetrameric complexes with rat anti-mouse Fc region antibodies (Wognum *et al.,* 1989). The chemical manipulation involves dissociation of two different antibodies and reassociation of the half molecules (Nisonoff and Rivers, 1961). It does not require cell fusion, the desired bispecific and trispecific antibodies can be made more quickly and the products are also comparatively easier to purify (Glennie *et al.*, 1987). A drawback of the chemical recombination method is protein denaturation and loss of antibody activity.

Allelic exclusion in B cell development ensures the synthesis of only one H and one L type of the immunoglobulin chain for eventual assembly into an IgG molecule with two identical antigen binding arms. This is also a characteristic of hybridomas derived by B cell fusion. However, when two hybridomas are fused to form quadromas, the resulting cell can co-dominantly express the H and L chains of both the parental hybridomas. Heterologous association of H chains and homologous or conservative assembly of the light chains to the parental H chain generates bsMAbs with two dissimilar antigen binding properties in one molecule (Suresh, 1986a and b).

The cell fusion techniques avoid some disadvantages of the chemical methods but still have some shortcomings: difficulty of fusing two parental hybridoma cells, low stability of the resulting cell lines, low yields of the desired bsMAbs and difficulty in purification of the bispecific molecules free of parental and other species. Cell fusion is labor intensive, time consuming, and requires parental cell lines to be mutated and made sensitive to a selection medium before they can be used (Milstein and Cuello, 1983; Staerz and Bevan, 1986; Lanzavecchia and Scheidegger, 1987; Wong and Colvin, 1987).

Several approaches for selecting quadroma cells have been developed. Parental hybridomas could be reverted to be HAT-sensitive by selecting mutants that lack hypoxanthine guanine phosphoribosyl transferase (HGPRT) fused with the immune spleen cells (Milstein and Cuello, 1983). Also, hybridoma cells lacking other markers such as thymidine kinase or adenosine phosphoribosyltransferase may be selected in the medium containing bromodeoxyuridine or 6-chloropurine, respectively. Alternatively, with the use of two distinct site-specific irreversible inhibitors of protein synthesis, such as emetine, actinomycin D, or iodoacetamide, the metabolic pathway of each of the two parental cell lines could be inhibited independently (Suresh *et al.*, 1986a). Furthermore, somatic hybrids can be selected by sorting out the heterofluorescent cells without relying on drug selection (Karawajew *et al.*, 1987; Koolwijk *et al.*, 1988), but it requires an expensive fluorence activation cell sorter.

Recombinant DNA methods have also been exploited to generate transfectants to secrete bsMAbs (Songsivilai *et al.*, 1989). Several groups have developed single chain bispecific antibodies (Holliger *et al.*, 1993 and Kranz *et al.*, 1995). In their studies, the heavy and light chains of immunoglobulin binding regions are linked by a (Gly-Gly-Gly-Gly-Ser)$_3$ linker and two single chain binding regions are themselves linked by yet another semi-rigid bridge peptide. Another elegant method takes advantage of the *jun* and *fos* peptide segments of the oncogen proteins. A murine (α-CD3) Fab'-*fos* and (α-Tac) Fab'-*jun* were individually expressed as homodimers in SP2/0 cells. When reduced, mixed and reoxidized, a preferential heterodimer association was seen due to the formation of a leucine zipper between the fos and jun peptides fused to the two Fab' regions. These provide improved methods of generating new types of cells secreting only bsMAbs.

The conventional fusion method involves PEG which acts as glue adhering to cell membranes. Electrofusion, however, induces cell hybridization by transient electric fields. An initial alternative current (AC) aligns the cells exposed to the electrical field and the current is then switched to a direct electric field (DC) to breakdown the cell membranes. The membrane breakdown is caused by a transmembrane potential difference. When the critical transmembrane potential difference was reached, membrane breakdown and pores were formed, leading to a cross-flow of cytoplasm and medium. The pores of adjacent cells might broaden and eventually cause the two cells to fuse. When the DC is removed, the remaining pores in the membrane of the new heterokaryon close, yielding a hybrid cell (Glassy *et al.*, 1988). Electrofusion has a number of advantages over the conventional methods of cell fusion (Schmitt *et al.*, 1989, Zimmermann and Vienken, 1982). It is an innocuous and nonchemical method that does not seem to alter the biological structure or function of the target cells. In addition, it is relatively easier to perform and much more efficient than the traditional chemical or biological fusion techniques. It can improve the fusion yields and hybridoma recovery by 100-fold (Chang *et al.*, 1989) in comparison to the widely used PEG-fusion method.

Microelectrofusion allows a very small number of cells (~1000 cells) involved in generation of the quadromas with relatively high frequency. Such a protocol could also be predictably adopted to generate human hybridomas with few B cells isolated from peripheral blood lymphocytes enriched by antigen specific panning or affinity microelectrofusion. The desired quadromas can be selected with no drug selection procedures due to immediate plating of cells at low density to avoid overgrowth of unfused cells, which could otherwise mask detection of any putative quadromas in the well. It was observed that with a concentration of 10^5 cells/mL or 1000 cells/10μL, proper cell to cell contact and orientation for cell fusion is achieved and the quadroma can be selected by ELISA. We have noted that optimum total number of cells for seeding would be 10 cells in each well of 96-well plate. These five quadromas were generated in a month and remained stable after the second reclone.

Two of these new quadromas, anti-biotin x anti-HRPO and anti-biotin x anti-squamous carcinoma, were developed and characterized for further studies. BsMAb (anti-biotin/anti-HRPO) has been employed as a universal immunoprobe for detecting biotinylated macromolecules including biotin pegylated liposomes. This will be elaborated in chapter 4. Another bsMAb (anti-biotin/anti-squamous carcinoma) has been used to generate tumor specific drug delivery system, which will be addressed in chapter 6.

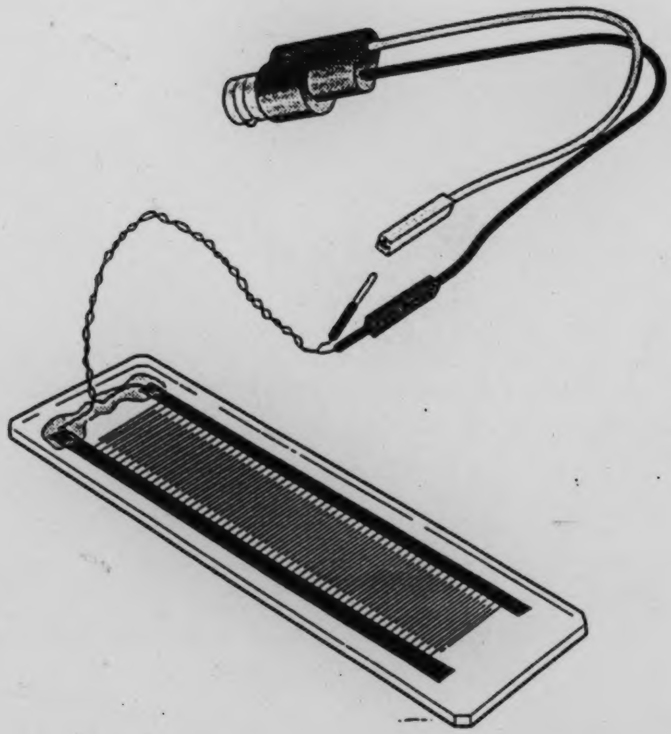


Figure 3.1 Diagramatic representation of a BTX meander chamber (reproduced with permission from BTX, San Diego).

Table 3.1 Screening Methods Employed for Identifying Various Quadromas Developed by Microelectrofusion.

	YP4 x anti-biotin	YP4 x 170	anti-biotin x 170	anti-biotin x 174	YP4 x 174
1. Antigen	biotin-BSA	idiotype antibody	idiotype antibody	idiotype antibody	idiotype antibody
2. Blocking	3% BSA	3% BSA	3% BSA	3% BSA	3% BSA
3. BsMAbs	anti-biotin x anti-HRP	anti-HRP x 170	anti-biotin x 170	anti-biotin x 174	anti-HRP x 174
4. Marker	HRP	HRP	biotin-HPR	biotin-HRP	HRP
5. Substrate	ABTS/H_2O_2	ABTS/H_2O_2	ABTS/H_2O_2	ABTS/H_2O_2	ABTS/H_2O_2

Table 3.2 Primary Screening of Quadromas Secreting Bispecific Antibodies.

	Clone 1	Clone 2	Clone 3	Clone 4	Clone 5	Clone 6	Clone 7	Clone 8	Clone 9
YP4 x 174	0.578								
α-biotin x 174	0.417	0.363	0.398	0.421					
α-biotin x 170	0.664	0.403	0.397	0.338	0.240	0.214			
YP4 x 174	0.466	0.356	0.32	0.321	0.306	0.295	0.284		
α-biotin x YP4	0.512	0.548	0.469	0.337	0.364	0.318	0.302	0.290	0.256

Data expressed as mean of the optical absorbance at 405 nm (n=3) after subtraction of the background. The standard deviation is less than 5% of the mean for all values.

Table 3.3 Summary of Results of the Different Microelectrofusion Experiments Generating Quadromas.

Cell type	Fusion Volume (μL)	No. Cells/μL	No. Wells Seeded	No. Cells Per Well	No. Clones	No. Positive Clones	Frequency (10^{-3}) [a]	Frequency (%) [b]	Stability (%) [c]
anti-biotin x YP4	5	100	192	5	6	1	1	16.7	100
	10	100	96	10	10	4	4	40	100
	20	100	96	20	24	4	2	16.7	75
	50	200	96	100	96	0	0	0	0
YP4 x 170	10	100	96	10	16	3	3	18.7	100
	10	500	96	50	28	4	1	14.3	75
anti-biotin x 170	10	100	96	10	19	3	3	15.8	66
	10	500	96	50	36	3	1	8.3	66
anti-biotin x 174	10	100	96	10	8	4	4	50	75
YP4 x 174	5	100	96	5	10	1	2	10	100

a Frequency: number of positive quadroma clones divided by total input cells.

b Frequency: number of positive quadroma clones divided by total growing clones.

Stability: number of quadroma clones after second reclone divided by number of total positive clones.

CHAPTER 4

DEVELOPMENT OF BSMAB (ANTI-BIOTIN X ANTI-HRPO) AS A UNIVERSAL IMMUNOPROBE FOR DETECTING BIOTINYLATED MACROMOLECULES

This chapter has been accepted by Journal of Immunological Methods for publication (1998)

1. INTRODUCTION

The advent of MAb technology was instrumental in the development of immunoenzymetric and radioimmunometric assays. However, the drawbacks of covalent cross-linking of antibodies to enzyme or radioactive markers often generate batch to batch variations in conjugate preparations and denature their activities (Suresh *et al.,* 1986a and b). BsMAb with two distinct binding specificities could theoretically solve this problem. They were first illustrated in 1960's (Nisonoff and Rivers, 1961). In the past decade, especially after hybridoma technology was fully developed (Kohler and Milstein, 1975), bsMAb was developed as a second generation probe for immunoassays (Kreutz and Suresh, 1995 and 1997), immunohistochemistry (Milstein and Cuello, 1983), cell targeting (Lanzavecchia and Scheidegger, 1987), complement-mediated cytotoxicity (Wong and Colvin, 1987), targeting of toxins or drugs to cells (Raso and Griffin, 1981) and molecular hybridization (Auriol *et al.,* 1994). BsMAb designed specifically to cross-link an antigen with an enzyme can eliminate the need for chemical conjugation and give a greater degree of linearity, sensitivity and reproducibility (Suresh *et al.,* 1986a and b).

In this chapter, employing a non-selective microelectrofusion, we have successfully developed and characterized a desired quadroma cell line P58.5R, which continuously secretes bsMAb with both anti-biotin and anti-HRPO binding specificities.

2. MATERIALS AND METHODS

2.1 PREPARATION OF BIOTINYLATED PROTEIN AND LIPOSOME

Chemical reagents used for preparation of antigens including d-biotin, keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), ovalbumin (OVA), NHS-LC-biotin [N-hydroxysuccinimidyl-6-(biotinamido) hexanoate], carbodiimide, aqueous pyridine, and 4-hydroxy-azobenzene-2'-carboxylic acid (HABA) were purchased from Sigma Chemical Company, Mississauga, ON, Canada. The hydrogenated soy phosphatidylcholine (HSPC), cholesterol (CH), biotin-phosphatidylethanolamine (B-PE) were purchased from Princeton Lipids (Princeton, NJ). Polyethylene glycol (MW 2000 Da) phospholipid distearoyl-phosphatidylethanolamine (PEG2000-DSPE) was a gift from Dr. T. Allen, Pharmacology Department, University of Alberta.

Biotin was chemically attached to KLH, BSA and OVA and the molar ratio of biotin to protein was determined by HABA assay (chapter 2). The liposomes were prepared using a method described previously (New, 1990). HSPC : Cholesterol (molar ratio 2:1) were mixed and dissolved in chloroform to create a homogeneous mixture. Biotinylated conventional liposomes were prepared by adding 2% biotin-PE to lipid mixture. Biotin stealth liposomes were prepared by adding 2, 5 and 10 mol % PEG-DSPE to lipid mixture. The mixture was placed in a round-bottom flask and the solvent was removed under negative pressure using a rotary evaporator. An evenly-distributed thin lipid film was formed on the flask wall and stored *in vacuum* overnight. The thin lipid film was hydrated with PBS (pH 7.4) to produce multilamellar liposomes. The homogeneously sized liposomes were obtained by extrusion (Lipex Biomembranes Extruder, Vancouver, BC. Canada) through polycarbonate membrane filters successively with 0.2, 0.1 and 0.08 μm pore size until the liposome size was about 100 nm. The liposome size was monitored by dynamic light scattering with a Particle Analyzer (Brookhaven Instruments Corporation, Holtsville, NY).

2.2. DEVELOPMENT AND CHARACTERIZATION OF BSMAB

2.2.1 Microelectrofusion

YC6/41, a rat hybridoma secreting IgG2a anti-horseradish peroxidase (anti-HRPO) MAb was kindly provided by Dr. Milstein from MRC Laboratory of Molecular Biology, U.K. P54.1R, a mouse hybridoma secreting IgG1 anti-biotin MAb was developed in our lab. The detailed fusion protocol was described as before (chapter 3). Clones appeared in about 10 to 14 days and the supernatants were tested for bsMAb production by a bridge ELISA method described below. The best quadroma P58.5R were recloned twice and stored in liquid N_2.

2.2.2 BsMAb Bridge ELISA

Standard 96-well ELISA plates were coated with 1 μg/well biotinylated BSA at room temperature overnight. The wells were blocked with 3% BSA/PBS (pH 7.4) for 2 h at room temperature. After washing 5 times with PBS/Tween-20 (0.02%), 50 μL of supernatant (1:100 dilution) mixed with 50 μL of HRPO at 10 μg/mL was added and incubated for 1 h at room temperature. This was followed by washing 5 times again, and color reaction was developed by ABTS + H_2O_2. The bsMAb is specifically detected in this assay format by its ability to bridge the solid phase biotin antigen with the solution phase HRPO.

2.2.3 Production and Dual Affinity Purification

Quadroma cells were grown in the culture medium RPMI-1640 with 5% FBS over 3 weeks. Two liters of supernatant were collected and centrifuged at 200x g for 7 min to remove cells. The supernatant was passed through a 10 mL biotin-agarose column (Sigma). The unbound antibodies were washed away by 200 mL of phosphate buffer (pH 7.4). The anti-biotin antibodies were eluted with excess antigen (0.5 mM d-biotin) or 0.2 M glycine buffer (pH 2.8) followed by immediate neutralization with phosphate buffer (pH 8.0). The fractions were dialyzed against PBS buffer (pH 7.4) to remove biotin. Antibodies were then purified by passing through a 10 mL HRPO-Sepharose column (Sigma). The column was eluted with 50 μg/mL HRPO or 0.2 M glycine buffer (pH 2.8) followed by neutralization to pH 7.4. The protein concentration and activity of each fraction were measured by UV (280 nm) and ELISA (405 nm) respectively. The purity was analyzed by reduced SDS-PAGE.

2.2.4 Determination of Binding Efficiency

a) Binding efficiency of P58.5R to biotinylated OVA was determined by competitive ELISA, making comparison to streptavidin-HRPO conjugate (Vincent and Samuel, 1993). Standard 96-well ELISA plates were coated with 1 μg/well biotinylated OVA in PBS (pH 7.4) at room temperature overnight. The plates were blocked with 3% BSA/PBS. 50 μL of serial dilution of biotinylated OVA was mixed with 50 μL of P58.5R/HRPO complex or streptavidin-HRPO conjugate at optimal dilution respectively. The final concentration of P58.5R was 1 μg/mL and HRPO was 10 μg/mL. The mixture was added to the pre-coated plates and incubated for 3 h at room temperature. After washing step, the plates were developed by ABTS + H_2O_2

b) Binding efficiency of P58.5R to d-biotin and biotinylated OVA was also determined by competitive ELISA described above.

2.2.5 Background Noise Determination

The background noise and specific binding of anti-biotin MAb P54.1R and bsMAb P58.5R to different antigens were performed and compared to avidin and streptavidin. Eight different antigens were included: biotin-KLH, biotin-BSA, biotin-OVA, KLH, BSA, OVA, normal mouse serum and normal human serum. Direct ELISA method was used as described above.

2.3. APPLICATIONS OF P58.5R IN DIFFERENT BIOASSAY SYSTEMS

2.3.1 Immunoassay

Standard 96-well ELISA plates were coated with 1 μg/well biotinylated OVA (80 pmole of free biotin) at room temperature overnight. The wells were blocked with 3% BSA/PBS (pH 7.4) for 2 h at room temperature. After washing 5 times with

PBS/Tween-20 (0.02%), 50 μL of different amount of purified P58.5R mixed with 50 μL of HRPO at 10 μg/mL was added and incubated for different time periods at room temperature. This was followed by washing 5 times again, and color reaction developed for different time points by ABTS + H_2O_2. To test the limits of sensitivity of the bsMAb P58.5R, biotinylated OVA was coated on a microtitre plate in serial dilution followed by ELISA.

2.3.2 Liposomal Immunosorbent Assay

The accessibility of biotin moiety of liposome to the anti-biotin MAb could be determined using a sandwich liposomal immunoassay (Fig 4.1, p 75). The 96-well microtiter plates were coated using purified 1 μg of anti-biotin monospecific MAb P54.1R as capture antibody overnight at 4^0C. The nonspecific binding sites were blocked with 3% BSA/PBS. The biotinylated liposomes of different preparations were added and incubated for 1 h at room temperature. The plates were washed 5 times by PBS/Tween (0.02%). 100 μL of bsMAb P58.5R/HRPO complex (P58.5R at 1 μg/mL and HRPO at 10 μg/mL) was added and incubated for 30 min at room temperature. Color was developed by adding ABTS + H_2O_2. The same concentration of P58.5R/HRPO complex was applied in all the subsequent assays.

2.3.3 Quantitative Measurement of Biotin

The determination of biotin concentration was performed by competitive enzyme immunoassay. Standard 96-well ELISA plates were coated with 1 μg/well biotinylated BSA in PBS (pH 7.4) as the solid phase capture reagent at room temperature overnight. The nonspecific binding sites were blocked by 200 μL of 3% BSA/PBS. The wells were washed with PBS/Tween-20 (0.02%). 50 μL bsMAb/HRPO complex mixed with 50 μL of standard biotin or biotinylated liposome solution at various concentrations were added for 2 h at room temperature. The color was developed by adding ABTS + H_2O_2.

2.3.4 Dot Blot and Western Blot

To detect biotinylated proteins on a western blot, biotinylated OVA was resolved on SDS-PAGE and transferred onto nitrocellulose paper. Alternatively, biotinylated OVA was spotted on nitrocellulose as 2-5 μL dot blot. The nitrocellulose was blocked with 3% BSA/PBS and incubated with P58.5R/HRPO for 1 h and washed. The location of biotinylated OVA was identified using 4-chloro-naphthol + H_2O_2 which generates a blue precipitate for a positive reaction.

3. RESULTS AND DISCUSSION

3.1. Development of BsMAb P58.5R

The development of a quadroma is time and labor intensive because the two parental hybridoma cell lines generally need a separate drug marker for selection. Hence a novel non-selective methodology was developed (chapter 3) and standardized to generate the desired quadroma secreting bispecific antibodies. This novel protocol was based on the micro-electrofusion on a meander chamber using a few hundred cells from each of the two parental hybridomas. This avoided laborious drug selection procedures. Quadromas could be selected without drug selection markers due to high cell fusion rate and subsequent limiting dilution. Nine primary quadroma clones were identified using the bsMAb bridge assay. The quadroma P58.5R was shown good stability and therefore was chosen for large scale production of bsMAb.

3.2. Purification and Characterization of BsMAb P58.5R

Quadromas co-dominantly express the two parental monospecific antibodies along with the desired bsMAb. Hence, we used dual affinity chromatography to remove the undesired monospecific MAbs (Fig 4.2, P 76). The raw supernatant was purified through both a biotin-agarose and a HRPO-Sepharose column. We use a competitive ligand (biotin or HRPO) elution (Harlow and Lane, 1988), making comparison to traditional acid elution. Fig. 4.3 shows the purification of bsMAb through biotin-agarose column with 0.5 mM biotin. In contrast, when acidic condition pH 2.8 was applied in a traditional acid elution, there was little or no recovery of antibody as evidenced by the UV absorption of the sample at 280 nm. This indicated that the antibody has a very high affinity for biotin and the acidic condition was not strong enough to break the interaction between antibody and antigen. The pooled MAbs eluted from the first biotin agarose column would have both bsMAb and monospecific anti-biotin MAb. Extensive dialysis of the pooled MAb could remove the biotin ligand from the antibodies. With the second HRPO-Sepharose column (data not shown), the similar results were observed with both HRPO competitive and acid elutions. However, the competitive elution would be preferred because it supplies mild condition to antibody and eluted bsMAb/HRPO complex could be directly used in ELISA without adding additional HRPO.

The biotin/avidin is widely employed in biochemical research, like nonisotopic immunoassay, nucleic acid hybridization assays and affinity chromatography due to the high affinity of the receptor-ligand interaction.(Diamandis and Christopoulos, 1991). Its success is attested to by a number of reviews (Wilchek and Bayer, 1984 and 1988). However, the disadvantage to the use of avidin is its tendency to bind nonspecifically to components other than biotin due to its high pI and carbohydrate content. The strong positive charge on the protein causes ionic interactions with more negatively charged molecules, especially cell surfaces. In addition, carbohydrate binding proteins on cells can interact with the polysaccharide portions on the avidin molecule to bind them in region devoid of targeted biotinylated molecules. These nonspecific interactions can lead to elevated background signals in some assays.

Streptavidin is another biotin binding protein isolated from *Streptomyces avidinii* that can overcome some of the nonspecificities of avidin (Chaiet and Wolf, 1964). Similar to avidin, streptavidin contains four subunits, each with a single biotin binding site. After some postsecretory modifications, the intact tetrameric protein has a molecular mass of about 60 kD, slightly less than that of avidin (Bayer, 1990). The primary structure of streptavidin is considerably different from that of avidin, despite the fact that they both bind biotin with similar avidity. This variation in the amino acid sequence results in a much lower isoelectric point for streptavidin (pI 5-6) than the highly basic pI of 10 for avidin. Streptavidin has relatively reduced nonspecific binding due to lack of carbohydrate and low isoelectric point (pI 5-6). However, background noise is still a problem and restricts the full utilization of this system (Riley and Caffrey, 1990; Wilchek and Bayer, 1988; Diamandis and Christopoulos, 1991).

The results of background noise determination shows that both monospecific MAb P54.1R and bsMAb P58.5R have no cross reactivity to BSA, OVA, KLH, normal mouse serum and normal human serum in contrast to avidin or streptavidin which exhibited slightly elevated background signal under the ELISA wash procedures (Table 4.1, p 85). In our experiments with the immunoassay format employing P54.1R and P58.5R, they give a clear low background compared to avidin or streptavidin.

In this study, bsMAb P58.5R offered several advantages over current avidin/streptavidin technology. First, P58.5R bypassed the need for chemical coupling with HRPO. This avoided the denaturation of antibody or enzyme, saved time and achieved optimal reproducibility. Second, P58.5R can give low background noise due to its high specificity. Third, P58.5R can be continuously supplied by an established quadroma. Fourth, P58.5R demonstrates similar binding efficiency to streptavidin-HRPO despite its lower intrinsic affinity to biotin when binding to biotinylated macromolecules (Fig. 4.4, p 78). We also found that 100-1000 fold more d-biotin is needed than biotinylated OVA to competitively inhibit the binding of bsMAb/HRPO. This can be explained on the hypothesis that the antibody binds better to biotin clusters than small free d-biotin molecules. This has been confirmed by another research group (Vincent and Samuel, 1993).

3.3 Applications of BsMAb P58.5R in Different Bioassay Systems

3.3.1 Immunoassay of Biotinylated Proteins.

The bsMAb P58.5R purified through dual affinity columns with a competitive elution showed a very high activity. This purified antibody was used to determine the optimal dilution, antibody/antigen reaction time and color developing time when detecting 1 μg of biotinylated OVA (80 pmole equivalents of free biotin) with 10 μg/mL HRPO. We found that 1 μg/mL of P58.5R with 10 min incubation and 20 min color developing can give a signal over 0.5 (Fig. 4.5, p 79). The detection limit of P58.5R was 0.8 pmole of biotin in an ELISA (Fig. 4.6, p 80). Because we assume that 100% of biotin groups get adsorbed on the microtiter plate surface, the detection limit is the conservative estimate. One of the hallmarks of our ELISA experiments with bsMAb was the low background compared to the corresponding values with secondary reagents such as goat anti-mouse HRPO conjugate or the HRPO conjugates of avidin or streptavidin.

3.3.2 Liposomal Immunosorbent Assay.

Biotinylated liposome provides a multivalent surface to facilitate numerous antigen/antibody interactions and thus enhances the avidity of a binding event (Plant *et al.*, 1989). Another advantage of biotinylated liposome is the enormous capacity of the vesicles that may deliver a large amount of enzymes, fluorescent probes, radiolabeled compounds or therapeutic molecules to the target and thus increase the sensitivity of an assay or efficacy of the treatment (Jones *et al.*, 1996).

We have established a sandwich immunoassay to quickly measure the characteristics of the various liposome preparations. Fig. 4.1 shows schematically the immunoassay format we developed incorporating a bsMAb P58.5R in a sandwich assay with a solid phase monospecific anti-biotin MAb P54.1R. Fig. 4.7 (p 81) shows that the biotin moieties of liposome with or without PEG can be strongly bound by the anti-biotin binding arm of P58.5R. However, liposomes with 10% PEG exhibited reduced binding to antibody compared to liposome with 0, 2% and 5% PEG. The steric hindrance of PEG on biotin accessibility for receptor binding has also been addressed previously (Mori *et al.*, 1991).

3.3.3 Quantitative Measurement of Biotin

Several methods exist for the determination of biotin concentration, including microbiological assays (Skeggs, 1966), spectrophotometric assays (Green, 1970), fluorescent polarization (Schray *et al.*, 1988), and isotope dilution (Yankofsky *et al.*, 1982). However, these methods are either less sensitive or time consuming. Recently, Chang and his colleagues developed a competitive ELISA for the determination of biotin concentrations using streptavidin-HRPO conjugate (Chang *et al.*, 1994). Here, we have proposed an alternative by using P58.5R/HRPO complex in stead of streptavidin-HRPO in this assay. Our strategy is based on the competition of binding to anti-biotin binding arm of bsMAb between solution and solid phase biotin.

When more biotin molecules were present initially in the reaction solution, less free bsMab/HRPO complex would be available to be immobilized by solid phase biotinylated BSA. Biotin concentration could be obtained from the standard biotin calibration curve. Fig. 4.8 shows the standard curve that could be used to measure the biotin concentration of the biotinylated liposome sample and free biotin.

3.3.4 Western Blot and Dot Blots

In addition to immunoassay, P58.5R was used in dot blots and western blots to detect biotinylated OVA. We found that detection limit in western blot (Fig 4.9, p 83) was 80 pmole of biotin. This was 100 times less sensitive than that achieved by ELISA. In nitrocellulose based DNA dot blots (Fig 4.10, p 84), P58.5R was shown to generate a blue product with biotinylated DNA.

3.3.5 Detection of Biotinylated DNA

Radioisotope labeled DNA or RNA probes are still widely applied for Northern blot, Southern blot and *in situ* hybridization techniques due to their high sensitivity and amplification effect for autoradiography. However, safety problems, reduced stability of radioactively labeled probes and long time needed for visualization have stimulated interest in the development of non-radioactive probes (Forster *et al.*, 1985). Several non-radioactive methods have been developed based on immunological detection of chemically modified nucleic acids, such as fluorochrome (Baumann, 1985), mercurated probes (Hopman *et al.*, 1986) and dinitrophenyl (Shroyer and Nakane, 1983).

The development of a biotin-avidin system and the successful synthesis of biotin-labeled (d) UTP, opened new prospects in non-radioactive detection of nucleic acids (Varma *et al.*, 1994). Another bsMAb anti-biotin x anti-HRPO was tested for its ability to detect biotin-DNA using luminescent substrates for HRPO followed by autoradio graphic detection (Leong *et al.*, 1986), but it was less sensitive than autoradiographic methods using ^{32}P-labeled probes. Recently, a digoxigenin anti-digoxigenin system was proposed and detection was mediated by high-affinity anti-digoxigenin antibodies conjugated to either alkaline phosphatase, peroxidase, fluorescein and rhodamine or revealed by a secondary antibody bound to colloidal gold (Li *et al.*, 1993). In nitrocellulose based DNA dot blot assays, our newly developed P58.5R with high affinity and lower background was shown to generate a blue product with biotinylated DNA obtained from a commercial source. Our limited study on this aspect of the application of the bsMAb showed the feasibility of detecting biotin-DNA. In principle, this could be applied to the Southern blots as well.

3.3.6 Other Applications

a) Biospecific interaction analysis (BIA, Pharmacia Biosensor) based on surface plasmon resonance is an advancing analytical technology for monitoring biomolecular interactions (Karlsson *et al.*, 1991). The bsMAb P58.5R demonstrated binding to the biotin immobilized on the gold biosensor chip used in this system. If this is incorporated in a biospecific interaction analysis (BIA), it could provide quantitative data of affinity and kinetic parameters of antibody/antigen interaction.

b) P58.5R has also shown the potential to react with M13 phage with biotin peptide mimic expressed on its tail in an immunoassay. Thus, it could be used to detect or select the specified M13 phage in phage library or detect the recombinant proteins with a biotin peptide tag similar to either c-myc or poly-histidine tails.

In conclusion, bsMAb P58.5R (anti-biotin x anti-HRPO) has been developed as a universal immunoprobe for detecting biotinylated macromolecules. Also this bsMAb has been used to characterize biotinylated liposome as a drug carrier.

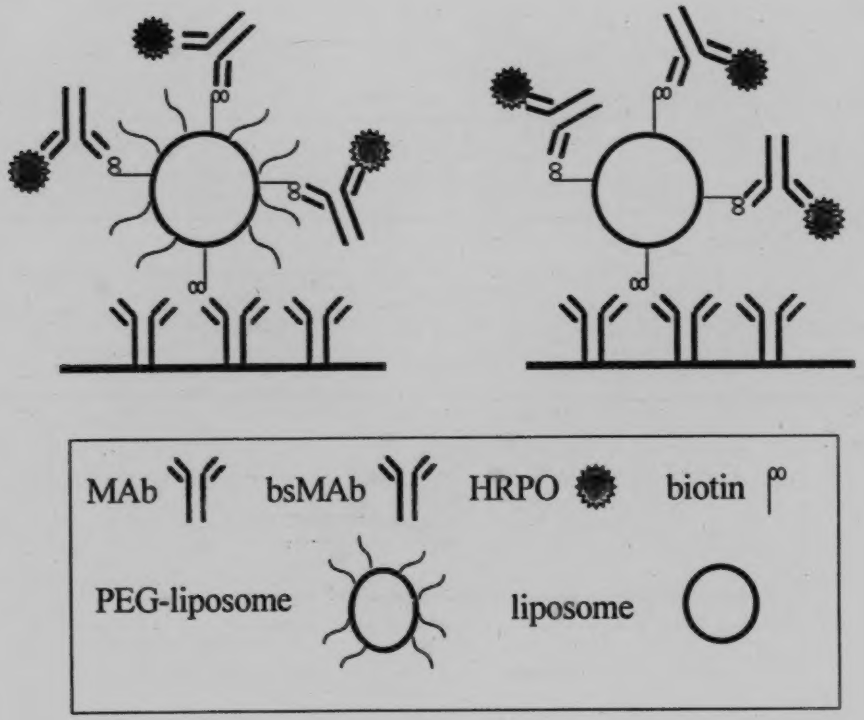

MAb
bsMAb
HRPO
biotin
PEG-liposome
liposome

Figure 4.1 Diagrammatic representation of sandwich immunoassay for detecting biotin moieties of biotin PEGylated liposomes.

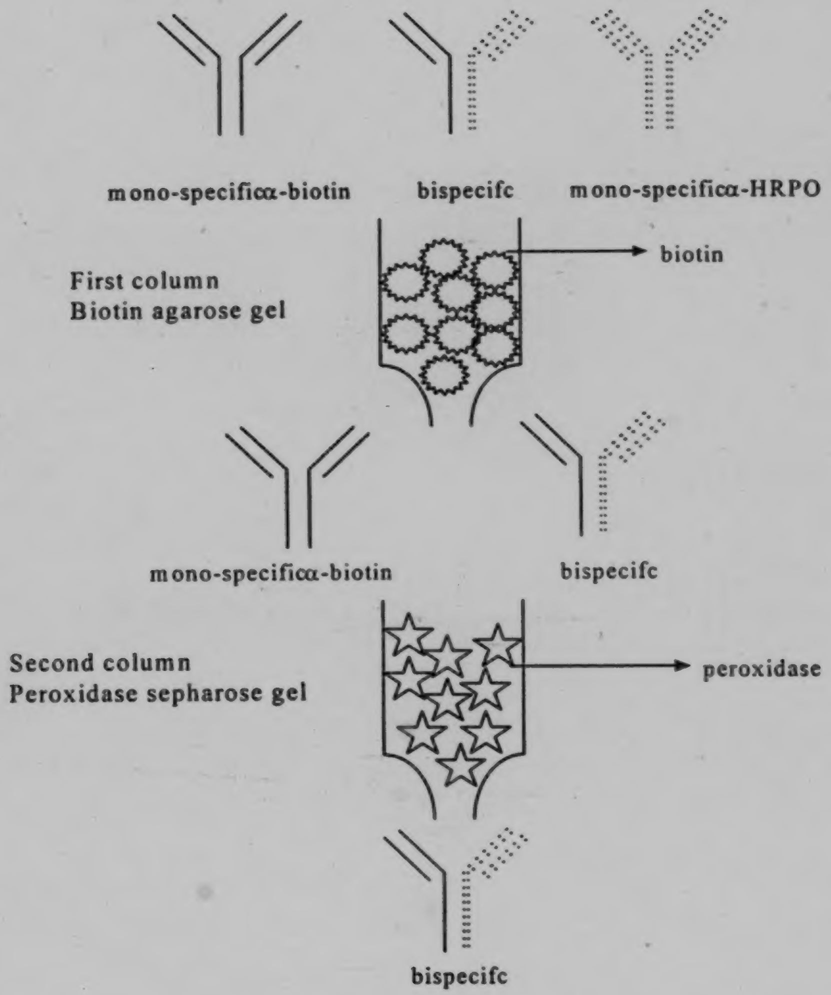

mono-specificα-biotin
bispecifc
mono-specificα-HRPO
First column
Biotin agarose gel
biotin
mono-specificα-biotin
bispecifc
Second column
Peroxidase sepharose gel
peroxidase
bispecifc

Figure 4.2 Schematic representation of dual affinity chromatography.

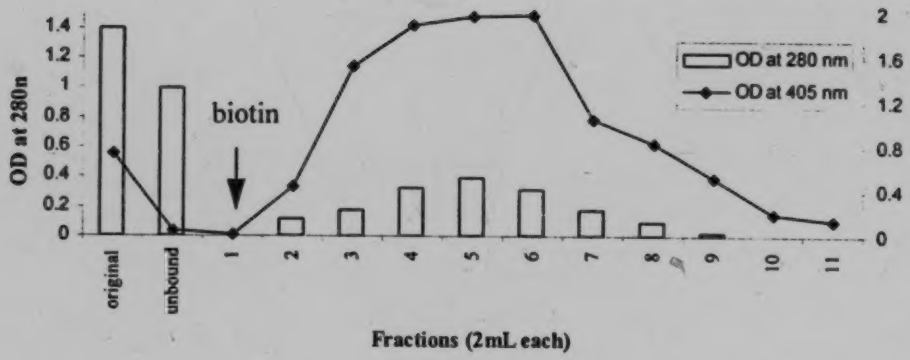


Figure 4.3 Purification of bsMAb P58 using biotin-agarose affinity column. The raw supernatant was loaded on a 10 mL biotin agarose column and washed with PBS. The bound antibodies were eluted with 0.5 mM biotin in PBS.

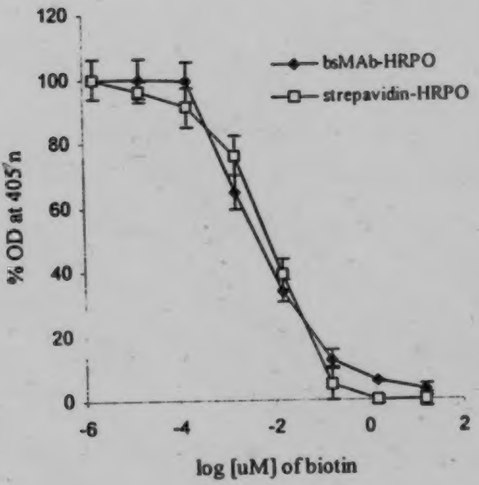


Figure 4.4 Relative binding efficiency of bsMAb vs. streptavidin-HRPO for biotinylated OVA. The solid phase antigen was biotinylated OVA coated on an ELISA plate. The concentration of biotin in biotinylated OVA has been calculated based on biotin to OVA molar ratio of 3.5.

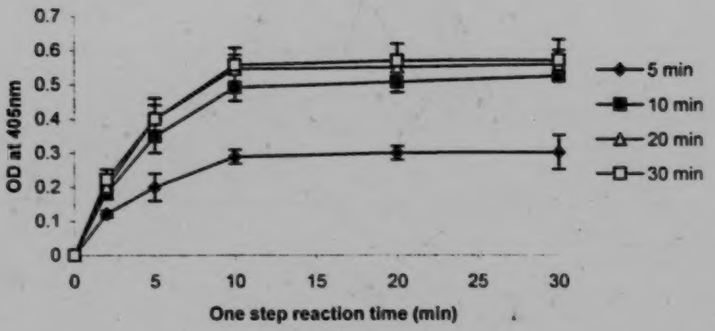


Figure 4.5 Effect of antigen-antibody reaction time and color developing time on the bsMAb ELISA. The purified bsMAb P58.5R (1 μg/ml) and 10 μg/mL HRPO were used. A 10 min incubation time was enough for the bsMAb bind to the solid phase biotin and 20 min was good for color development.

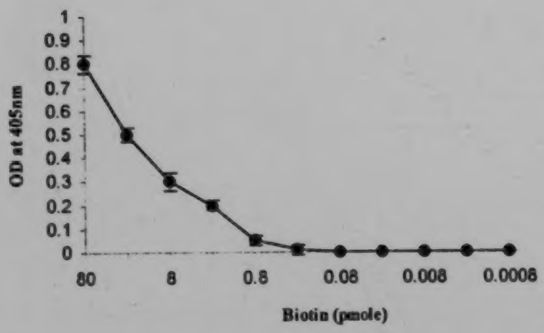
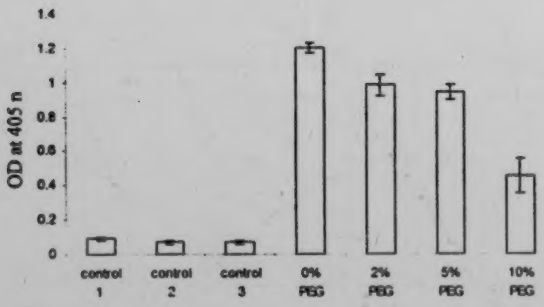


Figure 4.6 The limit of detection of biotin by bsMAb P58.5R in ELISA. The lowest limit detection is the least amount of biotin that can be detected with a predetermined confidence usually at 95%. It is defined as the value of biotin above the zero value signal plus 2 standard deviation. It appears to be 0.8 pmole as shown by serial dilution of the antigen, biotin-OVA.



Biotinylated liposome

Figure 4.7 Sandwich immunoassay for detecting biotinylated liposome with varying PEG concentration (n=4). Control-1: liposome without biotin, control 2: without adding biotinylated liposome; control-3: without bsMAb (anti-biotin X anti-HRPO).

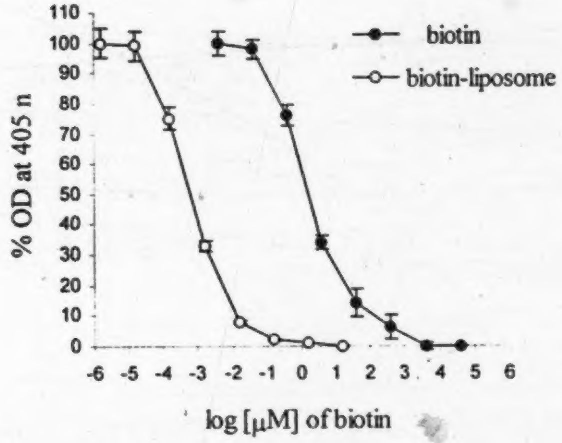


Figure 4.8 Determination of biotin concentration with competitive ELISA method. The P58.5R + HRPO complex was incubated with free biotin and biotin-liposome sample solution competing with solid phase biotin-BSA in an ELISA plate. Blocking and color development was performed as described in other figures.

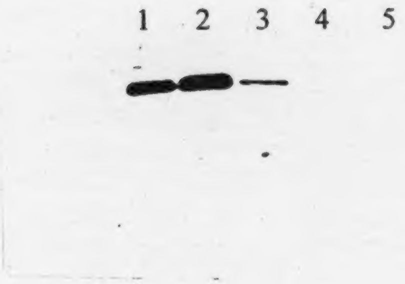


Figure 4.9 Western blot using bsMAb as the detection reagent for the different amount of biotinylated OVA. Lane 1: 10 μg; Lane 2: 1 μg; Lane 3: 0.1 μg; Lane 4: 0.01 μg. The negative control was 10 μg OVA.

10 μg	5 μg	1 μg	0.1 μg	0.01 μg	negative

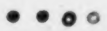


Figure 4.10 Dot blot using bsMAb as the detection reagent for the different amount of biotinylated OVA. Negative control was 10 μg OVA.

Table 4.1 Specific Binding Background Noise With Various Biotin Binding Macromolecules

	Biotin-KLH	Biotin-BSA	Biotin-OVA	KLH	BSA	OVA	Mouse[d] serum	Human serum
MAb P54.1R	++[a]	++	++	–	–[c]	–	–	–
BsMAb P58.5R	++	++	++	–	–	–	–	–
Avidin	++	++	++	+[b]	+	+	+	+
Streptavidin	++	++	++	+	+	+	+	+

[a] "++": mean optical absorbance at 405 nm higher than 0.3.

[b] "+": mean optical absorbance at 405 nm higher than 0.1 and lower than 0.5.

[c] "–": mean optical absorbance at 405 nm lower than 0.1.

The mean optical absorbance was taken from 4 values and the standard deviation was less than 5% of the mean.

[d] Mouse serum was collected from normal mouse without immunization

CHAPTER 5

A SIMPLE AND EFFICIENT METHOD FOR RADIOLABELING OF PREFORMED LIPOSOMES

Published in Journal of Pharmacy and Pharmaceutical Sciences 1, 1, 31-37, 1998

1. INTRODUCTION

Liposomes are artificial lipoid systems consisting of one or more phospholipid bilayers arranged concentrically with aqueous compartments and a core. They are made predominantly from naturally-occurring phospholipid and therefore they are biodegradable, innocuous and do not elicit an immune response. Liposomes were first introduced in the early 1960s by the British scientist Alec Bangham (Bangham, *et al.*, 1965). They have been employed as excellent models of biological membranes and are very useful carriers of both lipid-soluble and water-soluble drugs (Gregoriadis and Florence, 1993; Kim, 1994).

In recent years, the applications of these lipid spheres have been extended to enhance drug efficacy and potency (Mayer *et al.*, 1993 and 1995; Gabizon *et al.*, 1996), reduce toxicities of encapsulated drugs (Bally *et al.*, 1994; Rahman *et al.*, 1990; Lim *et al.*, 1997) and target specific tissue sites (Loughrey *et al.*, 1993a and b). In addition, liposomes which carry radionuclides within the aqueous space have potential diagnostic imaging applications (Lasic, 1992). Gamma-emitting radionuclides, such as ^{99m}Tc, ^{111}In and ^{67}Ga, have been encapsulated in liposomes for use as imaging agents (Gregoriadis *et al.*, 1977; Richardson *et al.*, 1978; Oku *et al.*, 1993; Tilcock *et al.*, 1993; Ogihara-Umeda *et al.*, 1994 and 1996). Some clinical trials for tumor imaging have already been started using radiolabeled liposomes (Presant *et al.*, 1988 and 1990; Kubo *et al.*, 1993). Furthermore, the radiolabel can be used to monitor pharmacokinetic and pharmacodynamic changes of drug-loaded liposome.

Generally, two methods have been used to radiolabel liposomes. One involves the entrapment of radionuclides during the hydration of the lipids with the aqueous phase (McDougall *et al.,* 1975). However, low encapsulation efficiency and batch production variability are two disadvantages of this approach. The second method is the labeling of preformed liposomes (Phillips *et al.,* 1992). Labeling of preformed liposomes with radionuclides is ideally suited for clinical imaging applications, wherein the liposomes can be formulated and distributed to the clinician as an unlabelled kit. This preparation is subsequently labeled on site with a short half-life radionuclide prior to clinical use.

In this chapter, I will describe the method for loading a model drug ^{99m}Tc-DISIDA (Hernandez and Rosenthall, 1980; Klingersmith *et al.,* 1981) to liposomes.

2. MATERIALS AND METHODS

2.1. Liposome Preparation

The hydrogenated soy phosphatidylcholine (HSPC), cholesterol and phospholipid distearoyl-phosphatidylethanolamine (DSPE) were purchased from Princeton Lipids (Princeton, NJ). The liposomes were prepared using the method described previously (chapter 4). HSPC : Cholesterol (molar ratio 2:1) were mixed and dissolved in organic solvent chloroform to create a homogeneous mixture. The mixture was placed in a round-bottom flask and the solvent was removed under negative pressure using a rotary evaporator. An evenly-distributed thin lipid film was formed on the flask wall and stored *in vacuo* overnight. The thin lipid film was rehydrated with 50 mM reduced glutathione (γ-Glu-Cys-Gly, Sigma, St Louis, Mo.) to produce multilamellar liposomes (MLV). Care was taken to disperse all of the dried lipid from the wall of the flask. The homogeneously sized liposomes were obtained by extrusion (Lipex Biomembranes Extruder, Vancouver, BC. Canada) through polycarbonate membrane filters successively with 0.2, 0.1 and 0.08 μm pore size until the average size distribution was ~100 nm in diameter. Removal of unencapsulated material was accomplished by ultracentrifugation (Kubo *et al.,* 1993). The liposome size was monitored by dynamic light scattering with a Particle Analyzer (Brookhaven Instruments Corporation, Holtsville, NY.). The liposome suspensions were diluted with PBS, then the sample was allowed to equilibrate for 15 min. at room temperature followed by size measurement at 25 °C. The concentration of the liposomes was determined by phosphorus content after perchloric acid treatment (Fiske and Subbarow, 1925).

2.2. Labeling Procedures

^{99m}Tc-DISIDA was purchased from the local Edmonton Radiopharmaceutical Center (ERC). The radiochemical purity was estimated by instant thin layer chromatography (ITLC) prior to use. ^{99m}Tc-DISIDA was incubated with glutathione encapsulated liposomes for 40 min. at room temperature. The labeled liposomes were separated from ^{99m}Tc-DISIDA by passage over a Sephadex G-25 column (1x15cm). Labeling efficiencies were checked by determining the activity before and after column separation of the ^{99m}Tc labeled liposomes using a dose calibrator.

2.3. Chloroform Extraction

The chloroform extraction was conducted with four different formulations: free ^{99m}Tc-DISIDA, ^{99m}Tc-DISIDA mixed with empty liposomes, ^{99m}Tc-DISIDA incubated with 50 mM glutathione for 40 min, and ^{99m}Tc-DISIDA incubated with glutathione loaded liposomes for 40 min.

A 0.1 mL aliquot of one of the different liposome formulations was added to 0.9 mL of saline in a 10 mL centrifuge tube, 1mL of chloroform was added to the sample and vortexed for 10 sec. The tube containing the mixture was centrifuged at 700x g for 5 min to separate the two phases. The lower layer (organic phase) was removed by using a glass syringe and transferred to a clean tube. The organic phase retained in the tube was washed with 1 mL of saline, vortexed, spun and separated from water phase as before. The washing step was repeated up to 3 times, retaining the organic phase each time. The final organic phase containing the total lipids of the sample was separated from water-soluble liposomal contents, and was counted using a Beckman 8000 gamma counter (New, 1990).

2.4. Stability of ^{99m}Tc Labeled Liposomes

(a) Gel filtration chromatography: ^{99m}Tc labeled liposomes were incubated in saline and 90% fetal bovine serum (FBS) at 37^{0}C for 24 h. A small sample of liposome was analyzed on a Sephadex G-25 column at different time points. The column was eluted with PBS and aliquots of the fractions were counted. A sample of ^{99m}Tc-DISIDA was also similarly analyzed to determine the difference in the separation profiles of the labeled compounds.

(b) Dialysis: A small volume of labeled liposomes in saline and 90% FBS, was placed in a dialysis sac (pore size 60 kD) and dialyzed against 100 mL of 0.9% saline at 37^{0}C for 24 h. Aliquots from the dialysates were taken at each time point and the radioactivity counted.

2.5. Biodistribution Studies

Ten week-old BALB/c mice were used for the biodistribution studies. ^{99m}Tc labeled liposomes (0.4 μmol as phospholipid and 5 μCi / 0.1 mL) and free ^{99m}Tc-DISIDA (5 μCi / 0.1 mL) were injected into the tail vein of normal mice. At 24 h postinjection, the BALB/c mice were anesthetized with Metofane and a blood sample was obtained by cardiac puncture prior to euthanasia. The organs were removed, thoroughly washed with saline, weighed and counted using a gamma counter. Biodistributions were calculated as percentage of the injected dose per gram and per organ. Total blood volume, bone, muscle, brain and skin mass were estimated as 5.4%, 10%, 40%, 0.7% and 13% of total body weight, respectively (Wu *et al.,* 1981).

3. RESULTS AND DISCUSSION

Radiolabeled liposomes have been successfully used to monitor pharmacokinetic changes of liposomes and image tumors, abscesses, ischemic and infarcted regions (Gabizon *et al.,* 1990; Goins *et al.,* 1993; Tilcock *et al.,* 1993; Ogihara-Umeda *et al.,* 1994 and 1996).

^{99m}Tc is the best candidate for imaging studies due to its short half-life, pure photon emitter and suitable energy. Encapsulating ^{99m}Tc during the rehydration of the lipid film with aqueous phase has been tried as a mothod to label liposomes (Oku *et al.*, 1993; Espinola *et al.*, 1979). This method is not convenient for clinical purposes due to individually-made preparations instead of batch-production and low incorporation efficiency (about 20-30%). Further, ^{99m}Tc has been used to directly label preformed liposomes based on using stannous chloride as a reducing agent (Love *et al.*, 1989). Reduced ^{99m}Tc is added to the liposomes and bound to the liposome outer surface. This surface-labeling approach is technically simple and good for the production of the vesicles in a pharmaceutically acceptable form. However, the bound ^{99m}Tc dissociates rapidly from the liposome surface *in vivo* by exchanging with plasma proteins and accumulates in the thyroid, stomach and kidney.

The lipophilic compound hexamethylpropyleneamine oxime (HMPAO) has been successfully used to label preformed liposomes (Goin *et al.*, 1993). This method has high labeling efficiency and stability because HMPAO, as a lipophilic chelator, can carry ^{99m}Tc inside preformed liposomes containing reduced glutathione. The liposome labeling mechanism is thought to be that ^{99m}Tc-HMPAO is chemically reduced in the presence of the glutathione, becoming more hydrophilic and therefore trapped within the liposomes. However, high cost limits the applications of the resulting imaging agent.

Efficient labeling of preformed liposomes was observed in our studies using ^{99m}Tc-DISIDA (Fig 5.1, p 94). ^{99m}Tc-labeled derivative of IDA (Iminodiacetic acid) was first introduced by Loberg (Loberg *et al.*, 1976) for use as a hepatobiliary imaging agent. Later, IDA derivatives like 2,6-diethyl, *p*-ethoxy, and *p*-iodo-IDA were developed and showed that all had excellent hepatobiliary specificity while differing in blood clearance and urinary excretion (Wistow *et al.*, 1977). ^{99m}Tc DISIDA is a newer derivative and has been shown to have better hepatobiliary characteristics (Hernandez and Rosenthall, 1980; Klingersmith *et al.*, 1981).

The organic component of ^{99m}Tc-DISIDA is a bifunctional chelate having both the properties of technetium binding (iminodiacetic structure) and hepatobiliary processing (aromatic system). The lipophilic properties of this compound was demonstrated in chloroform extraction studies (Table 5.1, p 98) and shown that more than 80% of the ^{99m}Tc-DISIDA was extracted into the organic phase from the aqueous phase with ^{99m}Tc-DISIDA alone or mixed with empty liposomes.

The tripeptide (γ-Glu-Cys-Gly) glutathione (MW 307.3 D) is involved in many aspects of metabolism, including removal of hydroperoxides, protection from ionizing radiation, maintenance of sulfhydryl status of proteins and aiding detoxification. Many of these functions are accomplished by reactions at the cysteinyl sulfhydryl group (Cooper, 1983). When glutathione was incubated with ^{99m}Tc-DISIDA, the ^{99m}Tc complex could undergo decomposition by reduction with cysteinyl sulfhydryl group of glutathione to become more hydrophilic species. This resulted in less than 30% of radioactivity extracted into organic phase from aqueous phase (Table 5.1). This could also be an explanation for trapping ^{99m}Tc inside of liposomes when glutathione was encapsulated in the inner aqueous phase of liposomes.

Chromatography profile of the labeled liposomes on a Sephadex G-25 column showed that almost 90% of the radioactivity was recovered in the void volume associated with the liposome fraction (Fig 5.2, p 95). However, ^{99m}Tc-DISIDA alone eluted much later when chromatographed separately.

The stability of ^{99m}Tc labeled liposomes were demonstrated by both resolution of the labeled liposomes through the Sephadex G-25 column and dialysis against saline at various time points of post-labeling (Fig 5.3, p 96). Only about 15% and 20% of the radioactivity was released over 24 h when labeled liposomes were incubated with 90% FBS and saline respectively. The retention difference of the labeled liposomes incubated with serum and saline is probably due to the binding of radioisotope to serum components which could not diffuse through the dialysis membrane (Love *et al.*, 1989).

Biodistribution studies obtained from tissue samples at 24 h post-injection of ^{99m}Tc labeled liposomes (Fig. 5.4, p 97 and Table 5.2, p 99) show that significant radioactivity was localized in the liver and spleen (8.4% and 12.2% dose/gram, respectively). However, biodistribution of free ^{99m}Tc-DISIDA show that only 0.2% and 0.09% dose/gram was localized in the liver and spleen. The statistical differences were significant by student's t-test ($P<0.01$). This is because the liver and spleen are two major organs of the reticuloendothelial system (RES) which are known to accumulate and metabolize liposomes.

However, ^{99m}Tc-DISIDA, used for imaging and functional studies of liver and bile, is processed by the hepatocytes within a few minutes after administration and excreted rapidly via the hepatobiliary system to the intestine (Saha, 1987). Therefore, after 24 h post-injection, there is much less radioactivity remaining in liver (0.2% dose/gram) and spleen (0.09% dose/gram). A significant difference ($p<0.01$) was also found in the blood pool due to longer circulation time of liposomes compared to rapid excretion of free ^{99m}Tc-DISIDA.

The conventional liposome formulations have a tendency to accumulate in the reticuloendothelial system and this has compromised their applications in drug delivery involving other tissues (Allen, 1994). Recently, liposomes exhibiting greatly prolonged blood retention were developed, by incorporating certain lipids, such as mono-sialo-ganglioside (G_{MI}) or synthetic lipids derivatized with the hydrophilic polymer polyethylene glycol, into the liposomal membrane (Woodle and Lasic, 1992). Improved therapeutic effect was observed using these long-circulating liposomes (Allen, 1994, Allen *et al.,* 1992). For tumor imaging, long-circulating liposomes may lead to high tumor accumulation due to their long circulation time (Woodle, 1993). Thus, the methodology described above could also be employed for labeling these long-circulating liposomes. In conclusion, the labeling technique presented here shows a practical alternative for monitoring pharmacokinetic changes of liposomes or liposomal derivatives, and could also be used for tumor or infection imaging, especially coupled with targeting antibodies.

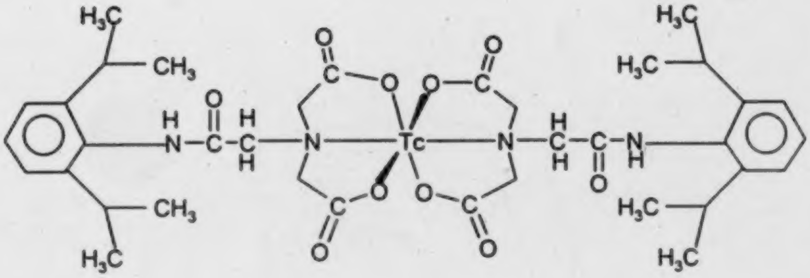


Figure 5.1 Structure of ^{99m}Tc-DISIDA complex (MW. 894 D).

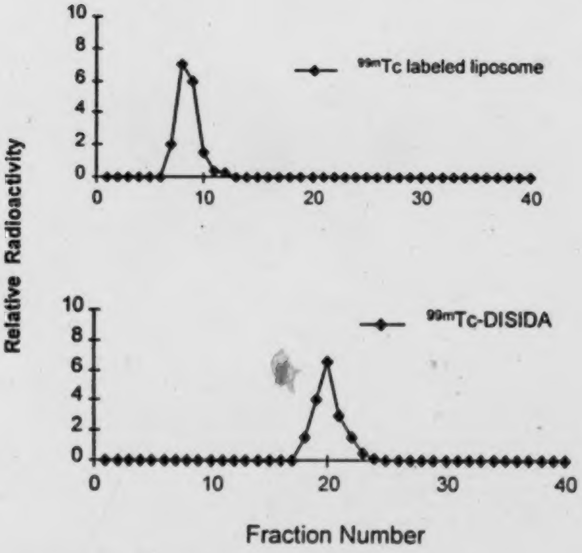


Figure 5.2 Sephadex G-25 column separation profile of ^{99m}Tc labeled liposomes and ^{99m}Tc-DISIDA alone.

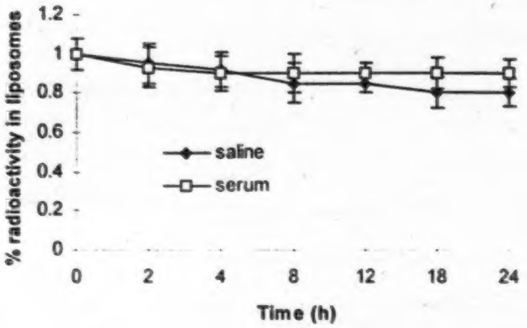


Figure 5.3 Retention of radioactivity in liposomes from dialysis of ^{99m}Tc labeled liposomes as a function of incubation time at 37 °C.

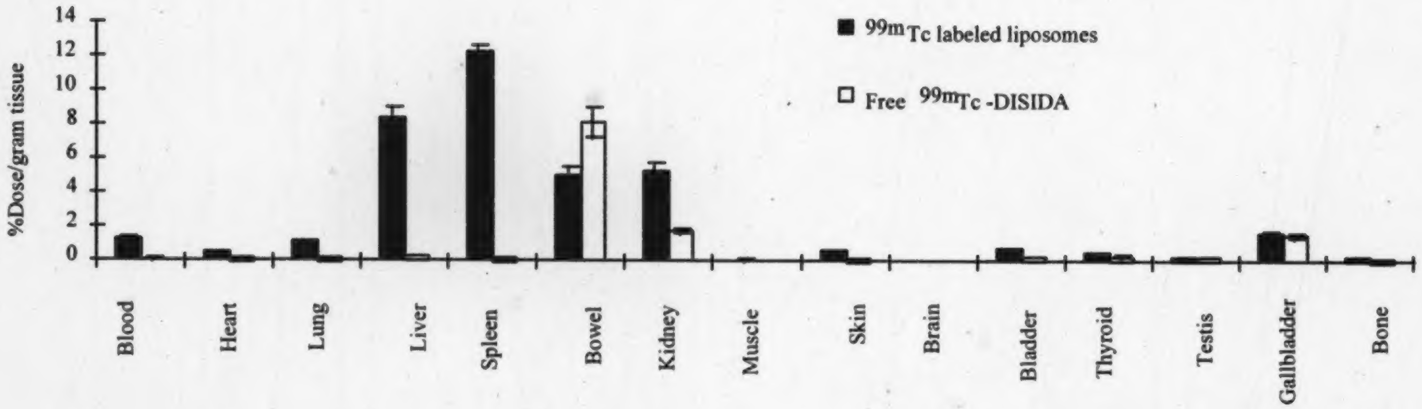


Figure 5.4 Biodistribution curve shows the difference of tissue uptake between ^{99m}Tc labeled liposomes and free ^{99m}Tc-DISDIA.

Table 5.1. Chloroform Extraction of Radioactivity from the Saline Phase of Different Formulation.

Formulation	% Radioactivity in Chloroform
1. ^{99m}Tc-DISIDA	82.3 ± 1.4
2. ^{99m}Tc-DISIDA + empty liposomes	81.7 ± 2.6
3. ^{99m}Tc-DISIDA + glutathione	28.3 ± 0.4
4. ^{99m}Tc-DISIDA + glutathione loaded liposomes	29.4 ± 0.6

Values are expressed as mean ± standard deviation (n=4).

Table 5.2 Tissue Biodistribution of ^{99m}Tc Labeled Liposomes at 24 H Post-Injection

Organ	^{99m}Tc-liposome (n=4)		^{99m}Tc-DISIDA (n=4)	
	%dose/organ	%dose/g	%dose/organ	%dose/g
Blood	2.10±0.15	1.25±0.08	0.10±0.01	0.06±0.01**
Heart	0.06±0.01	0.51±0.03	0.01±0.00	0.08±0.01**
Lungs	0.15±0.02	1.09±0.09	0.01±0.00	0.08±0.02**
Liver	11.72±0.8	8.4±0.58	0.27±0.01	0.2±0.01**
Spleen	2.66±1.45	12.21±0.42	0.02±0.00	0.09±0.01**
Bowel	11.98±1.23	4.98±0.52	20.34±1.94	8.13±0.92*
Kidney	2.01±0.19	5.31±0.43	0.49±0.05	1.75±0.14**
Muscle	0.07±0.01	0.06±0.01	0.03±0.01	0.02±0.00*
Skin	1.64±0.15	0.59±0.04	0.28±0.02	0.09±0.01*
Bone	0.47±0.06	0.23±0.00	0.19±0.02	0.11±0.01**
Brain	0.02±0.00	0.04 ±0.01	0.02±0.00	0.04±0.02
Bladder	0.03±0.00	0.75±0.06	0.01±0.00	0.26±0.02*
Thyroid	0.01±0.00	0.48±0.03	0.005±0.00	0.38±0.04
Gallbladder	0.02±0.00	0.06±0.01	0.05±0.01	0.19±0.07*

Data derived from mean ± standard deviation (n=4).

** t-test ($p<0.005$)

* t-test ($p<0.01$)

CHAPTER 6

ENHANCED TUMOR SPECIFIC DRUG DELIVERY BY PRETARGETING BISPECIFIC MAB AND BIOTIN PEGYLATED LIPOSOME

1. INTRODUCTION

Targeting drugs through a carrier system has been one of the main themes of research in therapeutics. The concept of drug targeting was introduced at the turn of the century by Paul Ehrlich (Ehrlich, 1906). Since the enunciation of this concept, antibodies have been developed and used for specific drug delivery to target tumor cells, primarily in the form of MAb-drug or MAb-toxin conjugates (Tyle and Ram, 1990). Two decades ago, liposomes were first proposed to be used for site-specific delivery of drugs (Gregoriadis, 1973). More recently, liposomal drug-delivery systems have come of age in oncology, with several liposomal drugs already licensed for use, e.g. Daunoxome® and Doxil® (Allen, 1997). Both preclinical and clinical studies have demonstrated that liposome-encapsulated drugs typically show reduced toxicities and augmented therapeutic efficacy compared to their non-liposomal counterparts (Ranson *et al.*, 1996). However, liposomes by themselves have no targeting capabilities except to the reticulo-endothelial system (RES).

Antibody covalently attached to classical liposomes (classical immunoliposomes) provides the vesicle an active targeting capacity that can specifically bind to antigenic determinants on cells. Immunoliposomes encapsulated with toxins or drugs can be used for tumor detection or therapy (Straubinger *et al.*, 1988; Matthay *et al.*, 1984; Heath *et al.*, 1983 and 1984, Allen and Moase, 1996). However, there are several limitations associated with the use of antibody-liposome conjugates for drug delivery.

First, the size of antibody conjugated liposomes are increased and their penetration to tumor targets may be more difficult than protein-free liposome, since their ability to pass through tissue barrier is limited by their size. Second, they are removed more quickly from blood circulation than liposome alone due to Fc-mediated clearance or protein-liposome aggregates (Debs *et al.*, 1987; Aragnol and Leserman 1986; Loughrey *et al.*, 1993). Lastly, the chemically coupling a liposome and a MAb is complex with aggregations and batch to batch variations in the preparation of immunoliposomes.

Polyethylene glycol (PEG) modification has changed the fate of liposome mediated tumor therapy to a certain extent. It stabilizes the liposome in circulation and reduces uptake of the liposome by the RES (Allen *et al.*, 1991; Senior *et al.*, 1991 and Papahadjopoulos *et al.*, 1991). It could allow potential therapeutic applications of immunoliposomes to be fully explored. Some experiments have shown that pegylated immunoliposomes, conjugated with an antibody against lung endothelial cells, would localize efficiently in murine lung tissues (Maruyama *et al.*, 1990 and Mori *et al.*, 1995). Further, the therapeutic application of pegylated immunoliposomes was demonstrated using doxorubicin entrapped in pegylated immunoliposomes linked to a monoclonal antibody to treat mice implanted with murine squamous lung carcinoma (Ahmad and Allen, 1992; Ahmad *et al.*, 1993; Allen *et al.*, 1995). The pegylated immunoliposome treatment appeared to be effective against early squamous lung carcinoma in mice. In addition to specific targeting, both internalization of immunoliposome-drug complex in those cells expressing an appropriate antigen with particular antibody and the 'bystander effect' on tumor cells lacking the specific epitope might contribute to the mechanism of cytotoxicity (Allen, 1994).

Alternatively, a two-step targeting approach has been explored for delivery of anti-cancer drugs loaded liposomes to tumor cells (Loughrey *et al.*, 1990 and 1993; Longman *et al.*, 1995). This approach consists of pretargeting tumor cells with biotinylated anti-tumor antibodies prior to administration of drug loaded liposomes that have streptavidin covalently attached to their surfaces. The problem of this strategy is the increased size of liposomes, immunogenicity of streptavidin and the effect of endogenous biotin on the normal tissue.

In this paper, we have established a novel promising new approach to combine bifunctional MAb and biotin pegylated liposome, which could overcome some of the major obstacles in the conventional strategies.

2. MATERIALS AND METHODS

2.1 Preparation of BsMAb P60 (174H.64 x anti-biotin)

174H.64 (Samuel *et al.*, 1989): a mouse hybridoma cell line secreting IgG1 anti-mouse squamous carcinoma antibody was kindly provided by Biomira Inc., Edmonton, Alberta, Canada. The P54.1R: a mouse hybridoma cell line secreting IgG1 anti-biotin antibody was developed in our lab. The detailed fusion protocol was described as before (chapter 3). Clones appeared in 10 to 14 days and the supernatants were tested for bispecific antibody production by an ELISA method described as below. Selected quadromas were recloned twice and stored in liquid N_2.

2.2 BsMAb Enzyme Immunoassay for Selecting Quadroma

Standard 96-well ELISA plates (Nunc, Naperville, IL) were coated with 1 μg/well anti-idiotype 174H.64 antibody at 4^0C overnight. The wells were blocked with 200 μL of 3% BSA/PBS (pH 7.4) overnight at 4°C. The wells were washed 5 times with PBS/Tween-20 (0.02%). Then 50 μL of supernatant (1:100 dilution) mixed with 50 μL of biotinylated HRPO at 10 μg/mL was added and incubated for 2 h at room temperature. This was followed by washing 5 times with PBS/Tween-20 (0.02%). Color reaction was developed by ABTS + H_2O_2 and plates were read after 30 min in a microplate reader (Molecular Devices Corporation, Sunnyvale, CA) at 405 nm.

2.3 Production and Purification

Male BALB/c mice were primed with 0.5 mL of Pristine (2, 6, 10, 14-tetramethylpentadecane) by intraperitoneal injection (Harlow and Lane, 1988). After two weeks, the monoclonal quadroma cells P60 1 x 10^6 in 0.2 mL saline were inoculated peritoneally into BALB/c mice. Ascites fluids were collected in tubes containing heparin two weeks after inoculation. The MAbs from the ascites fluids were diluted by PBS and purified by ammonium precipitation and ion exchange column. Briefly, solid ammonium sulfate was gradually added with stirring to achieve 50% salt saturation and the solution was continuously stirred at 4^0C overnight. The solution was centrifuged at 3000 x *g* for 30 min and the pellet collected. The precipitated immunoglobulins were dissolved in 10 mM sodium phosphate buffer (pH 7.5) and dialyzed against 4 L of the same buffer. The dialyzed sample was loaded on to a DE52 column equilibrated with 10 mM sodium phosphate buffer (pH 7.5). The column was washed to remove the unbound protein until no protein could be detected in the eluent. The immunoglobulins were eluted using a linear ionic gradient from 10 mM to 100 mM sodium phosphate buffer. The protein concentration was measured by UV detector at 280 nm. The bsMAb activity was determined by ELISA as described above. The fractions with highest antibody activity were pooled and the purity was analyzed by SDS-PAGE (Laemmli, 1970).

2.4 Preparation of Biotin-Pegylated Liposome and Biotin Conventional Liposome

The hydrogenated soy phosphatidylcholine (HSPC), cholesterol (CH), biotin-phosphatidylethanolamine (B-PE) were purchased from Princeton Lipids (Princeton, NJ). Polyethylene glycol (MW 2000 Da) phospholipid distearoyl-phosphatidylethanolamine (PEG2000-DSPE) were gifts from Dr. Allen, Pharmacology Department, University of Alberta. The liposomes were prepared using the method described previously (New, 1990). HSPC : Cholesterol (molar ratio 2:1) were mixed and dissolved in organic solvent chloroform to create a homogeneous mixture. Biotinylated conventional liposomes were prepared by adding varying amount of biotin-PE to lipid mixture. Biotin Pegylated liposomes were prepared by adding 2, 5 and 10 mol % PEG-DSPE to lipid mixture. The mixture was placed in a round-bottom flask and the solvent was removed under negative pressure using a rotary evaporator. An evenly-distributed thin lipid film was formed on the flask wall and stored *in vacuo* overnight. The thin lipid film was hydrated with PBS (pH 7.4) to produce multilamellar liposomes. The homogeneously sized liposomes were obtained by extrusion (Lipex Biomembranes Extruder, Vancouver, BC. Canada) through polycarbonate membrane filters successively with 0.2, 0.1 and 0.08 μm pore size. The liposome size was monitored by dynamic light scattering with a Particle Analyzer (Brookhaven Instruments Corporation, Holtsville, NY). The concentration of the liposomes was determined by phosphorus content after perchloric acid treatment (Fiske and Subbarow, 1925).

2.5 Sandwich Immunoassay for Detecting Biotin Moiety of Biotin Pegylated Liposome

The accessibility of the biotin moiety to the antibody could be determined using sandwich immunoassay (Fig 4.1, p 75). P54, MAb (anti-biotin) and P58, bsMAb (anti-biotin X anti-HRPO) were developed and characterized in our lab (Cao and Suresh, manuscript in preparation). The 96-well microtiter plates were coated using purified 1 μg of anti-biotin MAb as captive antibody overnight at 4^0C. The nonspecific binding sites were blocked with 3% BSA/PBS. The biotinylated liposomes of different preparations were added and incubated for 1 h at room temperature. The plates were washed 5 times with PBS/Tween (0.02%). Equal volumes of bsMAb (anti-biotin x anti-HRPO) and HRPO were added and incubated for 1 h at room temperature and washed. Color was developed by adding ABTS + H_2O_2.

2.6 Agglutination Studies by Streptavidin

Agglutination of biotin Pegylated liposomes by streptavidin was monitored by turbidity measurements (Mori *et al.*, 1991). 10 μL of streptavidin at different concentrations were added to 400 μL of liposome preparations (50 nmol as phospholipid) in PBS buffer (pH 7.4). The absorbance at 440 nm was monitored continuously.

2.7 Relative Avidity of BsMAb with D-Biotin and Biotin-Pegylated Liposome

The relative binding efficiency of anti-biotin binding site of bsMAb (174H.64 x anti-biotin) with d-biotin and biotin Pegylated liposome was evaluated by competitive enzyme immunoassay (Vincent and Samuel, 1993). Standard 96-well ELISA plates were coated with 1 μg/well biotin-BSA in PBS (pH 7.4) as the solid phase capture reagent at room temperature overnight. The nonspecific binding sites were blocked by 200 μL of 3% BSA/PBS. The wells were washed with PBS/Tween-20 (0.02%). 50 μL bsMAb at concentration of 10 μg/mL mixed with 50 μL of different dilutions of d-biotin or biotin-Pegylated liposome were added for 4 hr at room temperature. After washing 5 times with PBS/Tween buffer, 100 μL goat anti-mouse antibody conjugated with peroxidase at 1:1000 were added and incubated for 1 h at room temperature. The color was developed by adding ABTS + H_2O_2 for 30 min.

2.8 Loading of Model Drug ^{99m}Tc –DTPA Into Biotin-Pegylated Liposome

^{99m}Tc -DTPA was purchased from Edmonton Radiopharmaceutical Center, Edmonton, Alberta, Canada. The radiochemical purity was tested by instant thin layer chromatography (ITLC) before use. The loading method was described as before (Oku *et al.*, 1993). The thin lipid film was hydrated with 2 mL of ^{99m}Tc-DTPA solution with 0.5 mCi. After the extrusion process, the free ^{99m}Tc-DTPA was removed by passage over a Sephadex G-25 column (1 x 15 cm). The entrapping efficiencies were determined by the activity before and after column separation. Also, chromatography on silica gel paper developed in 0.9% saline was performed on the pre- and post-column samples. With this technique, the radiolabeled liposomes remain at the origin, while both free ^{99m}Tc and ^{99m}Tc-DTPA move with the solvent front.

2.9 Stability of Liposome Loaded With ^{99m}Tc-DTPA *In Vitro*

Liposomes loaded with ^{99m}Tc-DTPA were incubated in a water bath, with normal saline or 100% fetal bovine serum (FBS) at 37^0C for 24 h. A small sample of liposome was taken to pass through Sephadex G-25 column (Pharmacia, Quebec, Canada) at different time points. The column was eluted with PBS and fractions of eluent were counted in a gamma counter (Beckman Gamma 8000 series). An aliquot of $^{99m}TcO_4^-$ and ^{99m}Tc-DTPA were also used to determine the difference in the separation profiles of the compounds.

2.10 Targeting of Biotin Pegylated Liposome to KLN-205 cells by BsMAb *in vitro*

The murine squamous carcinoma cell line KLN205 was obtained from Biomira Inc. and grown in standard medium (RPMI 1640 supplemented with 10% FBS) in sterile microtitre plates strips. When the plates were confluent with tumor cells, the supernatant was removed and nonspecific binding sites were blocked by 3% BSA/PBS. BsMAb (174H.64 X anti-biotin) at varying concentrations were added and incubated for 1 h at room temperature. After washing twice, cells were incubated with biotin Pegylated liposome loaded with ^{99m}Tc-DTPA at varying concentrations for 1 h at room temperature. The wells were washed twice and radioactivity associated with the wells was measured in a gamma counter. HRPO as a solid phase capture antigen plus bsMAb (anti-biotin x anti-HRPO) was used as a positive control. PBS and monospecific anti-biotin MAbs instead of bsMAbs were used as negative controls.

2.11 Biodistribution Studies on Mice Bearing KLN-205 Allografts

The biodistribution studies were conducted in tumor bearing DBA/2 mice with four mice in each group. The strategy is shown in Figure 6.1 (p 114). Approximately $5x10^5$ KLN-205 cells were injected subcutaneously into 6-8 weeks old DBA/2 mice. After 2 weeks when the tumor grew to about 0.3-0.5 cm diameter, 50 μg of purified bsMAb (174H.64 x anti-biotin) was given *i.v.* The control used monospecific anti-biotin MAb. After 1 day, 50 nmol of multilamellar empty biotin liposome was injected to clear circulating antibodies. After 60 min, biotin pegylated liposome loaded with ^{99m}Tc-DTPA (0.5 μmol as phospholipid and 5 μCi in total volume of 0.1 mL) was injected into the tail vein of the experimental mice. 6 h after injection, the DBA/2 mice were anesthetized with metafane and a blood sample was obtained by cardiac puncture prior to euthanasia by cervical dislocation. Tissues were dissected from the carcass, The organs were removed, blotted to remove adhering blood, weighed and radioactivity measured in a gamma counter. 0.1 mL of labeled liposomes were placed in a plastic cuvette as the standard reference.

The final data were routinely expressed as either percent of the injected dose per gram or per organ. Total blood volume, bone, muscle, brain and skin mass were estimated as 5.4%, 10%, 40%, 0.7% and 13% of total body weight, respectively (Wu *et al.*, 1981). Values were expressed as mean ± SD, statistical analyses were done by the student t-test.

2.12 Effect of Streptavidin *in vivo* to Reduce the Blood Background

The biodistribution studies were conducted again in KLN-205 tumor bearing DBA/2 mice with four mice in each group as described above. The mice were injected *i.v.* with 50 μg purified bsMAb (174H.64 x anti-biotin) along with an appropriate control group using anti-biotin MAb. After 1 day, 50 nmol of MLV empty biotin liposome was injected to clear circulating bsMAb. After 60 min, biotin pegylated liposomes loaded with ^{99m}Tc-DTPA (0.5 μmol as phospholipid and 5 μCi in total volume of 0.1 mL) were injected into the tail vein of the experimental mice. 6 h after injection, 30 μg streptavidin was administered *i.v.* to the study group. After 1 h, biodistribution was done on the DBA/2 mice described as before.

3. RESULTS AND DISCUSSION

3.1 Preparation of BsMAb P60 (174H.64 x anti-biotin)

The hybridoma cell line 174H.64 secretes a murine MAb, which selectively recognizes an extra-cellular surface component of the cytoskeletal matrix system ectopically expressed on the proliferative compartment of mammalian squamous cell carcinomas (Samuel, *et al.*, 1989). In view of the insoluble nature of the antigen and the difficulty in obtaining large amounts of the same antigen for testing the immunoreactivity of the MAb 174H.64, an anti-idiotype ELISA assay for potency determinations was established (Suresh *et al.*, unpublished data). This assay is highly specific to the MAb 174H.64 variable region and is not reactive with other classes or subclasses of matched murine MAbs. A mouse hybridoma cell line P54.1R that secretes monospecific monoclonal antibodies against biotin was developed and characterized in our lab (chapter 2). This is an alternative probe to detect biotin residues in macromolecules to the traditional avidin and streptavidin method (Diamandis and Christopoulos, 1991). The hybridoma was used to generate secondary quadromas that secrete bsMAbs bearing an anti-biotin binding arm.

A novel non-selective methodology was developed (chapter 3) and standardized to generate quadromas, which secrete bsMAb anti-squamous carcinoma x anti-biotin. The generated quadromas were selected by bispecific immunoassay with 174H.64 anti-idiotype antibody as a capture reagent. Several quadroma clones were identified using the bsMAb bridge assay and the best one was recloned twice for monoclonality by limiting dilution. The mouse ascites was used for a large scale production of bsMAb and purified by ion exchange chromatography DE-52 column (data not shown). The purity was analyzed by reduced SDS-PAGE. This bsMAb was used to develop our new drug delivery system.

3.2 Preparation of Biotin Pegylated Liposome

The extruded vesicles prepared for this study had the following mean sizes: small unilamellar vesicle biotin Pegylated liposome (SUV): 101 ± 8 nm; biotin-liposome (SUV): 97 ± 9 nm; biotin PEG-liposome entrapped with ^{99m}Tc-DTPA (SUV): 92 ± 10 nm; multilamellar vesicle biotin liposome (MLV): 417 ± 33 nm. Biotin liposome can provide a multi-valent surface to facilitate numerous antigen/antibody interactions and thus enhances the avidity of a binding event. Plant and coworkers (Plant *et al.*, 1989) used liposomes containing biotinylated phospholipid components in a liposome immunosorbant assay with anti-biotin antibody or streptavidin molecule, creating large multivalent complexes able to bind antigen. This format has been confirmed and extended in our studies. Figure 4.1 (p. 75) shows schematically the immunoassay format we developed incorporating a bsMAb in a sandwich assay with a monospecific anti-biotin MAb. This was a convenient way to quickly measure the characteristics of the various liposome preparations including the influence of PEG and amount of biotin incorporated in the vesicle. Figure 6.2 (p 115) shows that biotin pegylated liposome appears to have 1000 to 10,000-fold more binding avidity compared to free biotin hapten when binding to anti-biotin binding arm of bsMAb. This could minimize the influence of endogenous biotin which is the potential problem in this approach.

Another advantage of this system is the huge capacity of the vesicles that may deliver a large amount of enzymes, fluorescent probes, radiolabeled compounds or therapeutic molecules to the target and thus increases the sensitivity of an assay or efficacy of the treatment. Jones and his colleagues (Jones *et al.*, 1993, 1994 and 1996) found that one small liposome could provides up to 10^5 molecules of fluorophore or radiolabeled moieties to allow excellent detectability of a binding event. Therefore, this system using biotinylated liposomes to detect antigen molecules can increase the sensitivity of an immunoassay up to 100-fold than that using traditional antibody-enzyme ELISA.

Biotinylated liposomes usually are created by modification of phosphatidylethanolamine components with an amine-reactive biotin derivative, such as NHS-LC-Biotin. The NHS ester reacts with the primary amine of phosphatidylethanolamine (PE) residues, forming an amide bond linkage. Since the modification occurs at the hydrophilic end of the phospholipid molecule, after vesicle formation the biotin component protrudes from the liposomal surface. In this configuration, the surface-immobilized biotin molecules are able to bind anti-biotin antibody or streptavidin molecules present in the outer aqueous medium of the liposome membrane. Using biotinylated phospholipid allows incorporation of discrete amounts of biotin binding sites into the final liposomal membrane.

Pegylated liposomes have shown the ability to localize in high concentrations at solid tumors (Allen, 1994). The mechanism involved could be due to three factors: (1) an increased permeability of tumor vasculature during the process of angiogenesis that would allow liposomes to escape the leaky capillaries, (2) a prolonged circulation time and reduced uptake by RES that would enable them to have enough time to pass the capillaries and reach the tumor sites and (3) enhanced lubrication properties of pegylated liposomes (vascular permeability coefficient) enable them to transit more easily across capillaries to reach the tumor interstitial space (Allen, 1994; Papahadjopoulos *et al.*, 1991; Gabizon *et al.*, 1996 and Huang, *et al.*, 1992). However, PEG polymers could have an effect on the antigen-antibody interactions on the liposomal surface. The results from sandwich immunoassy, shown in Figure 4.7, have demonstrated that the biotin moieties of both liposome and pegylated liposome can be strongly bound by the anti-biotin binding arm of bsMAb. However, liposomes with 10% PEG exhibited reduced binding to antibody compared to liposome without polymer or liposome with 2% and 5% PEG. This potential steric hindrance effect of PEG polymers at high concentration on biotin has also been addressed previously by (Mori *et al.*, 1991). The agglutination study (Fig 6.3, p 116) confirms that the biotin moieties on the surface of pegylated liposome could be accessed also by biotin binding proteins such as streptavidin. Since there are four binding sites in streptavidin, at the optimized concentration, the biotin pegylated liposomes might be clumped together to form large aggregates. This large complex *in vivo* would be cleared from blood circulation rapidly by RES and thus increase the tumor to blood ratio. Further, an additional benefit is to minimize the risk of damage to some critical organs like bone marrow.

^{99m}Tc-DTPA was used as a model drug for the study of drug specific targeting and delivery. The entrapping efficiency of ^{99m}Tc-DTPA-liposome determined by Sephadex G-25 column separation was about 30±4 %. The post-column preparations were used immediately for injection with a radiochemical purity >90%. The stability of ^{99m}Tc-DTPA loaded liposomes was tested by a small sample incubated with saline or 100% FBS at 37^{0}C for 24 h. Aliquots of the incubation mixture were assessed for free radiolabel using a Sephadex G-25 column at different time points. The column was eluted with PBS and fractions of eluent were measured in a gamma counter for radioactivity. The results were also confirmed by ITLC. Figure 6.4 (p 117) shows that about 20% of radiolabel was released from liposome incubated in saline compared to 30% released in FBS over 24 h ($P<0.001$). The difference might be due to the possibility that lipid proteins in FBS could promote the dissociation of the lipids making up the liposome.

3.3 Establishment of Specific Drug Delivery System

MAb 174H.64 of the IgG1 subclass with specificity for a cell surface marker expressed by more than 90% of squamous carcinomas was chosen as the target molecule. The marker belongs to the class of cytokeratins and is expressed by well-differentiated cells of the stem cell population of normal stratified epithelium and all squamous cell carcinomas. KLN-205 is a murine lung squamous carcinoma cell line reactive with MAb 174H.64 (Samuel *et al.*, 1989). This animal model has been successfully exploited in targeted drug delivery *in vitro* and *in vivo* (Ding *et al.*, 1990; Ahmad and Allen, 1992; Ahmad *et al.*, 1993).

Figure 6.5 (p 118) shows that bsMAb (174H.64 X anti-biotin), incorporating anti-biotin binding arm and anti-tumor binding arm, could bridge drug loaded liposomes to KLN-205 squamous cancer cells grown *in vitro*. This binding was nearly quantitative particularly at higher bsMAb concentrations, prompting us to test the model *in vivo*.

The tissue biodistribution (Table 6.1, p 121) in mice bearing KLN-205 squamous tumors shows that at the intervals of 6 h post-injection of pegylated liposomes with entrapped ^{99m}Tc-DTPA, the radioactivity in tumor was increased by pretargeting bsMAb (3.61% dose/g) compared to pegylated liposomes alone (0.89% dose/g, $p<0.01$). In the pretargeting strategy, several parameters should be considered. Baxter et al. (1992) concluded that 24 h is enough for the bsMAb to achieve sufficient concentration in the tumor and longer time delays are detrimental due to physiological considerations such as metabolism, tumor cell internalization and antigen shedding.

However, the intact bsMAb would result in low tumor to blood ratio due to its slow blood clearance. This could be overcome by the addition of a clearing agent, which could improve tumor to blood ratios with minimal change in the degree of tumor localization of liposome loaded drugs (Baxter *et al.,* 1992). In this approach, 24 h was chosen for the time interval between first two steps and multilamellar large biotinylated liposome (~400 nm) was used as a clearing agent since it could strongly bind to the bsMAb and be removed rapidly by RES.

In figure 6.6 (p 119), we can observe that ^{99m}Tc-DTPA localized in tumor were marginally increased from 2 h to 6 h of post-injection time and decreased afterwards. The blood and liver activity progressively decreased with time. Figure 6.7 (p 120)shows that ^{99m}Tc-DTPA loaded liposomes could be removed from blood pool to liver after adding 30 µg streptavidin. In our targeting strategy, this step 4 could be used optionally to enhance tumor to blood ratio.

This strategy developed here needs to be further optimized with varying time, bsMAb and biotin Pegylated liposomes. However it appears to have some conceptual advantages over other targeting strategies. (1) Pegylated liposomes used as drug carriers could deliver a large amount of radioisotopes or anti-cancer drugs to the target compared to antibody-drug conjugates or drugs alone; (2) Pretargeted bsMAb could minimize the limitations of immunoliposome applications *in vivo*, which has a short circulation time and fast clearance contributed by conjugated antibody molecules; (3) Modification of liposomal surface by biotin is a relatively easy process, unlike antibody coupling and would not affect liposome characteristics; (4) Multiple biotin moieties on the surface of pegylated liposome enable it to have a much higher local biotin concentration compared to free biotin hapten when binding to anti-biotin binding arm of bsMAb. This could minimize the influence of endogenous biotin which is the potential problem in this approach; (5) Since most of the cancers are heterogeneous, a nearby cancer cell that lacks the targeted epitope can also take up the released drugs from pegylated liposome and be killed (Allen, 1994). (6) Cross-linking multi-epitopes on the surface of a tumor cell could initiate endocytosis and intracellular delivery of cytotoxic drugs.

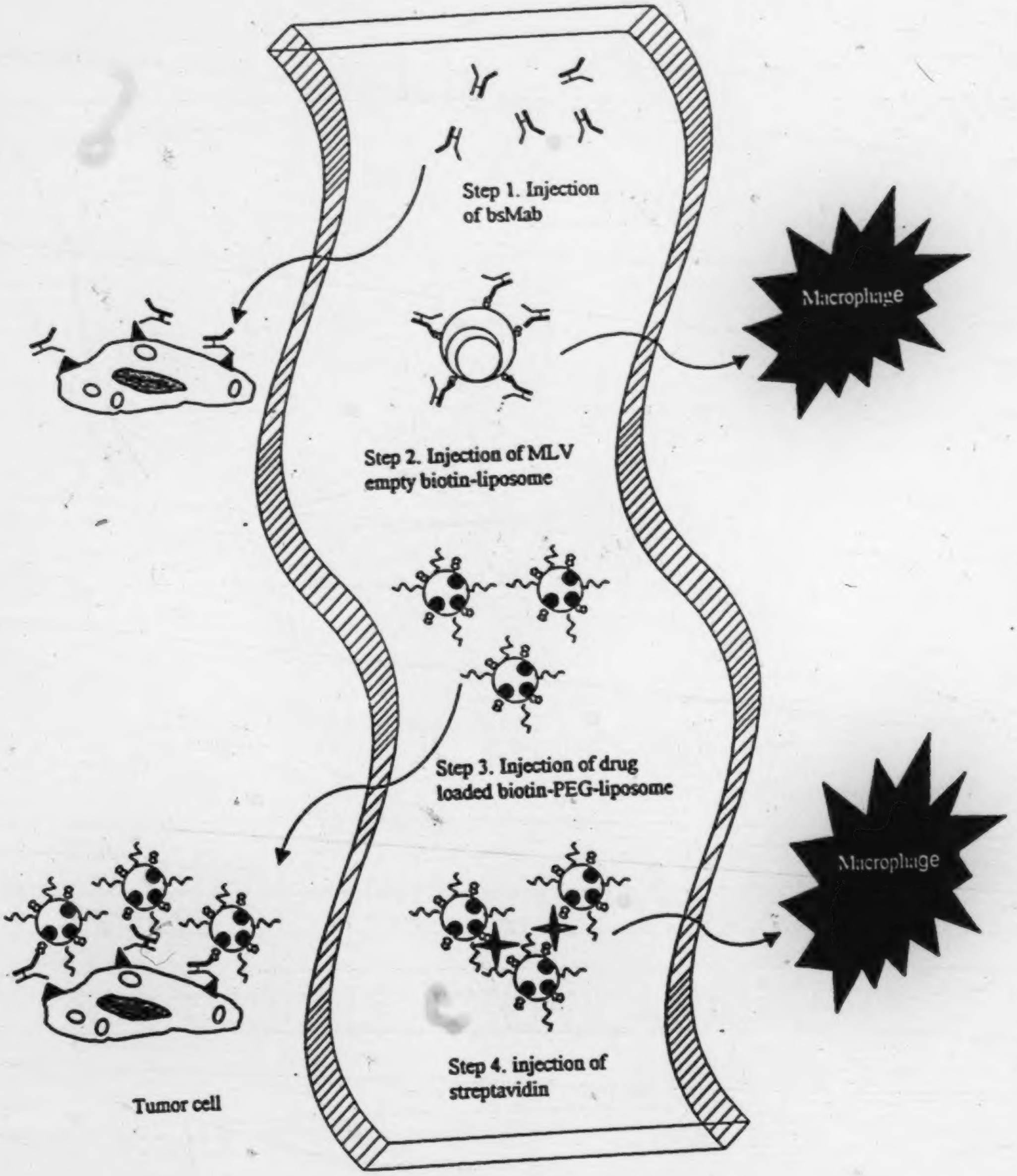

Step 1. Injection of bsMab
Macrophage
Step 2. Injection of MLV empty biotin-liposome
Step 3. Injection of drug loaded biotin-PEG-liposome
Macrophage
Tumor cell
Step 4. injection of streptavidin

Figure 6.1 Schematic representation of strategy for anti-cancer drug delivery.

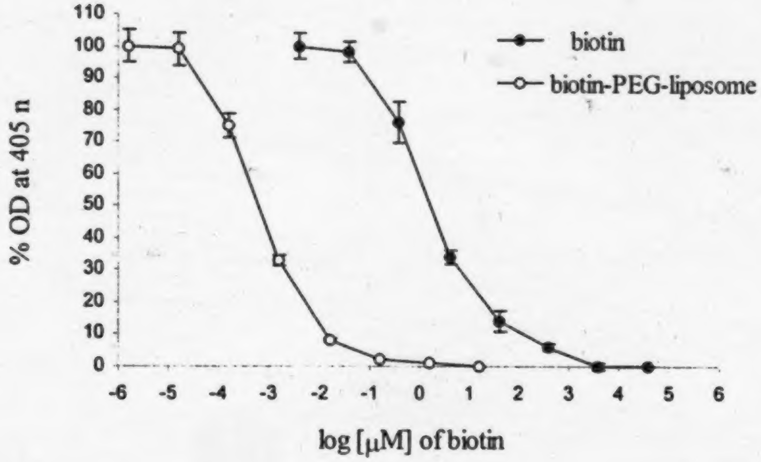


Figure 6.2 Relative binding avidity of bsMAb (174H.64 X anti-biotin) with biotin and biotin PEGylated liposome. Biotin and biotin PEGylated liposome in the solution could bind to bsMAb competing with solid phase coated biotin-BSA. The antibodies binding to the solid phase antigen were detected by goat anti-mouse IgG conjugated with HRPO. Relative binding avidity was determined by the concentration of competitor, which reaches 50% inhibition of bsMAb binding to the solid phase antigen due to the competitive antigen in the solution.

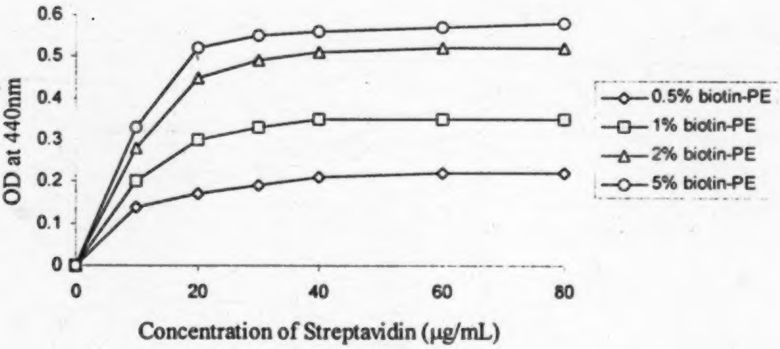


Figure 6.3 Effect of biotin content on the agglutination of biotin PEGylated liposome induced by streptavidin. Streptavidin mediated liposome turbidity was measured at 440 nm. Increasing the biotin content in liposome could enhance the turbidity of liposome. 2% biotin-PE and 20 μg/mL streptavidin were used for further study.

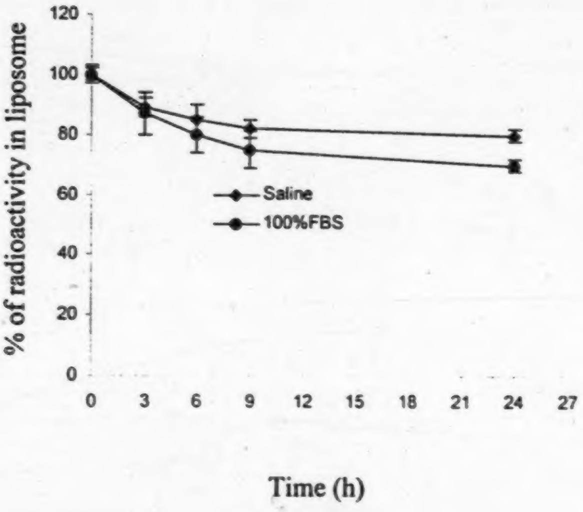


Figure 6.4 Stability of ^{99m}Tc-DTPA loaded biotin PEGylated liposome incubated with normal saline and 100% FBS at 37^{0}C for 24 h. After 24 h, about 20% of radiolabels was released from liposome incubated in saline and 30% released in FBS. The difference might be due to the lipid protein in FBS, which can prompt the dissociation of lipids from the liposome.

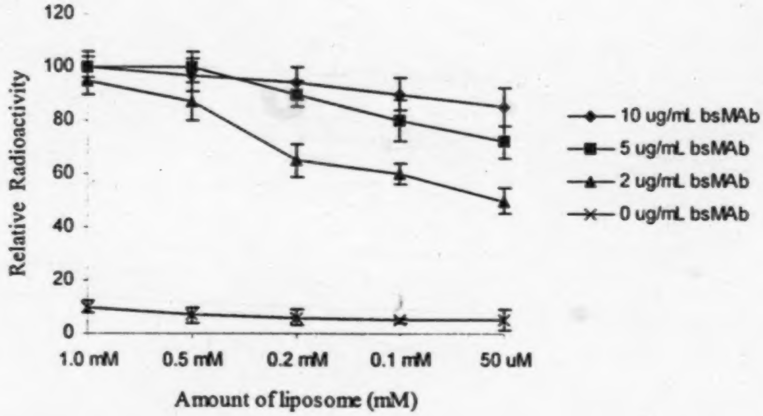


Figure 6.5 Targeting of biotin PEGylated liposome loaded with ^{99m}Tc-DTPA to KLN-205 cells by bsMAb (174H.64 X anti-biotin) *in vitro*. Data was expressed as relative radioactivity associated with tumor cells. Different amount of bsMAb and liposome were tested to optimize the targeting efficiency. At low bsMAb concentration (2 μg/mL), the amount of liposome targeted to the solid phase cancer cells are decreased significantly compared to the near quantitative binding at 5 and 10 μg/mL of bsMAb.

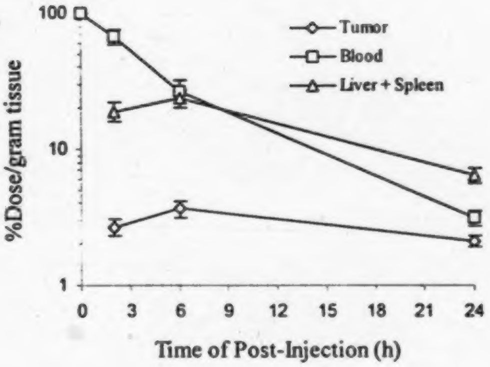


Figure 6.6 Biodistribution of ^{99m}Tc-DTPA loaded liposomes at different post-injection times. Data was expressed as percentage of injected does per gram of tissue (n=4).

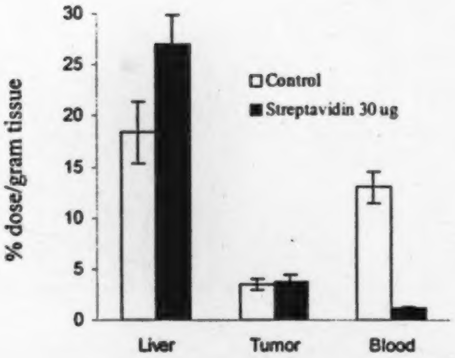


Figure 6.7 Effect of injection of streptavidin on the tissue biodistribution (n=4). The radioactivity was reduced sharply from blood pool to liver due to the agglutination effect of administration of streptavidin.

Table 6.1. Tissue Biodistribution of ^{99m}Tc-DTPA Loaded Liposomes at 6 H Post-Injection After 24 h Pretargeted with BsMAb.

Organ	Control group (n=4)		Study group (n=4)	
	%dose/organ	%dose/g	%dose/organ	%dose/g
Blood	29.5±1.64	22.8±1.24	28.7±1.36	21.6±1.22
Heart	0.09±0.01	0.81±0.09	0.09±0.01	0.76±0.1
Lungs	0.15±0.02	1.19±0.09	0.12±0.01	1.02±0.10
Liver	19.7±1.06	18.4±1.58	22.1±2.14	20.6±1.95
Spleen	1.96±0.15	18.21±1.93	1.67±0.12	17.6±1.85
Stomach	1.15±0.12	4.98±0.52	1.05±0.12	4.68±0.34
Kidney	2.03±0.19	4.31±0.43	1.73±0.2	4.33±0.46
Muscle	0.61±0.01	0.06±0.01	0.53±0.01	0.05±0.01
Tumor	0.22±0.02	0.89±0.08	0.90±0.01**	3.61±0.40**
Brain	0.01±0.00	0.07±0.01	0.01±0.00	0.08±0.01
Bladder	0.01±0.01	0.25±0.06	0.01±0.00	0.26±0.02
Thyroid	0.01±0.00	0.36±0.03	0.01±0.00	0.31±0.02
Gallbladder	0.04±0.01	1.52±0.14	0.05±0.01	1.49±0.13
Bone Marrow	0.17±0.06	0.23±0.03	0.19±0.02	0.21±0.02

The tissue biodistribution was performed at 6 h post-injection of ^{99m}Tc-DTPA loaded biotin PEGylated liposome. The study group of mice were pre-injected with 50 μg bsMAb (174H.64 X anti-biotin) 1 day ahead and the circulating antibodies in blood pool were cleared by adding 50 nmol of empty biotin-liposome (MLV, ~400 nm) for 1 h before giving the model drugs. The control group was treated the same as the study group except that pretargeted MAb was the monospecific anti-biotin MAb instead of bsMAb. ** $p<0.01$ by student's t-test.

SUMMARY AND FUTURE WORKS

Bispecific MAbs are unique versatile heterobifunctional cross-linkers capable of uniformly binding any two antigens in a pre-determined order. In this thesis, some new methods of rapidly generating hybrid-hybridomas was explored and the characteristics and applications of two bsMAbs, one in diagnostics and the other in therapeutics were studied. The common feature of both bsMAbs was the anti-biotin paratope. At the very onset, the hybridoma secreting anti-biotin MAb was developed using classical hybridoma technology. Initially, a hybridoma producing anti-biotin antibody was generated by fusion of myeloma SP 2/0 with the splenocytes collected from the mouse immunized with biotinylated KLH. The hybridoma P54.1R was chosen to further develop quadromas due to good stability and highest relative affinity. The anti-biotin MAb P54.1R was produced from mouse ascites, purified by affinity chromatography and characterized. The anti-biotin MAb P54.1R is an IgG1 (γ, λ) and has a binding affinity of 6.1×10^{-9} M to biotin.

A micro-electrofusion method was developed to generate desired quadromas secreting bsMAbs. This novel protocol is based on the microelectrofusion on a meander chamber using a few hundred cells of each of the two parental hybridomas without involving drug selection procedures. Quadromas could be selected by fusing any two hybridomas without drug involvement due to high cell fusion rate and subsequent limiting dilution. Such a protocol could also be predictably adopted to generate human hybridomas with few B cells isolated from peripheral blood lymphocytes enriched by antigen specific panning or affinity microelectrofusion. Thus, the development of this new method accelerated bsMAb development in our lab and in particular it allowed me to characterize and study two bsMAbs in detail.

The quadroma P58.5R producing bsMAb (anti-biotin x anti-HRPO) was developed by fusion of a hybridoma P54.1R (anti-biotin) with a hybridoma YP4 (anti-HRPO). The quadroma was recloned, expanded and stored in liquid N_2. The highly pure bsMAb was obtained with dual affinity column method. The purified anti-biotin x anti-HRPO bsMAb has been used to detect many different macromolecules bearing biotin moieties with high sensitivity and low background. The ELISA developed from this bsMAb is a single step procedure and gives a low background compared to similar tests using avidin/streptavidin. In this studies, bsMAb P58.5R can offer several advantages over current avidin/streptavidin technology. First, P58.5R can bypass the need for chemical coupling with HRPO. This will avoid the denaturation of antibody or enzyme, save time and achieve optimal reproducibility. Second, P58.5R results in a low background due to its high specificity. Third, P58.5R can be continuously supplied by an established quadroma. Fourth, P58.5R demonstrates similar binding efficiency to streptavidin-HRPO when binding to biotinylated macromolecules due to its high binding avidity. The bsMAb was successfully shown to bind biotinylated macromolecules such as protein, nucleic acid, biosensor surface or liposome. The ability of this universal biotin probe to bind even peptide mimics of biotin was a surprising result. Potentially, this bsMAb can be used to select recombinant proteins with biotin mimics as tags similar to the popular c-myc or histidine tags.

In the next aspect of this research, bsMAbs with anti-tumor and anti-biotin arms were exploited to target biotinylated liposomes *in vitro* and *in vivo*. Prior to achieving this goal, I had to develop a single method to load the model drug ^{99m}Tc-DISIDA into liposome. ^{99m}Tc was used as a model drug since it was convenient to measure. The labeling technique presented here shows a practical alternative for monitoring pharmacokinetic and pharmacodynamic changes of liposomes or liposomal derivatives.

BsMAb (174H.64 X anti-biotin) was developed to target drug loaded biotin pegylated liposomes to KLN-205 squamous cancer cells. In this study, bsMAb was injected 24 h ahead to pretarget the tumors with its anti-tumor epitope. MLV biotin-liposome without PEG (~400 nm) was used as a clearing reagent to remove circulating bsMAb to the RES. Subsequently, ^{99m}Tc-DTPA loaded biotin pegylated liposomes were injected and accumulated in tumor recognizing anti-biotin epitope of bsMAb. In addition, function of streptavidin as a clearing agent in the optional step was also evaluated. The tissue biodistribution in mice bearing KLN-205 squamous tumors shows that at 6 h of post-injection of liposomes with entrapped ^{99m}Tc-DTPA, the radioactivity in tumor was increased by pretargeting bsMAb (3.61% dose/g) compared to liposomes alone (0.89% dose/g, $p<0.01$). Figure 6.6 shows that drugs localized in tumor were increased from 2 h to 6 h of post-injection time and were afterwards. Figure 6.7 shows that the drug loaded liposomes could be removed from blood pool to liver after injecting 30 µg streptavidin as a chase step. In this targeting strategy, this step 4 could be used optionally to enhance tumor to blood ratio.

This new strategy has various advantages: (1) Pegylated liposomes have enormous capacity to deliver a large amount of radioisotopes or anti-cancer drugs to the target comparing to antibody-drug conjugates or drugs alone; (2) The limitations of immunoliposome are avoided which include a short circulation time and fast clearance contributed by conjugated antibody molecules. In addition, chemically linked antibodies or fragments are complicated procedures and may render the immunoliposome more immunogenic; (3) Modification of liposomal surface by biotin is a relatively easy process and would not affect liposome characteristics; (4) Multiple biotin moieties on the surface of pegylated liposome enable it to have much higher binding avidity than free biotin hapten (found in serum) when binding to anti-biotin binding arm of bsMAb. This could minimize the influence of endogenous biotin which is a potential problem in this approach; (5) Since most of cancers are heterogeneous, a nearby cancer cell that lacks the targeted epitope can also take up the released drugs from pegylated liposome and be killed. (6) Cross-linking multi-epitopes on the surface of a tumor cell could initiate endocytosis and intracellular delivery of cytotoxic drugs.

While initial attempts at bsMAb based pretargeting were made, additional optimization of the various parameters should be considered. (1) pegylated liposome with biotin at the chain end of PEG should be used. It could theoretically reduce the steric hindrance effect of PEG on the binding of biotin and anti-biotin MAb; (2) bispecific single chain antibody (bsFv) or diabodies should be used instead of whole antibody molecule. It would potentially increase the tumor targeting and minimize the immunogenic responses likely encountered with whole bsMAbs.

REFERENCES

Abra, R.M. and Hunt, C.A. (1981) Liposome disposition *in vivo*, dose and vesicle size. Biochim Biophys Acta 660, 493-503.

Affleck, K. and Embleton, M.J. (1992). Monoclonal antibody targeting of methotrexate (MTX) against MTX-resistant tumour cell lines. Br J Cancer 65, 838-44.

Ahmad, I. and Allen, T.M. (1992) Antibody-targeted specific binding and cytotoxicity of liposome-entrapped doxorubicin to lung cancer cells in vitro. Cancer Res 52, 4817-20.

Ahmad, I., Longenecker, M., Samuel, J. and Allen, T.M. (1993) Antibody-targeted delivery of doxorubicin entrapped in sterically stabilized liposomes can eradicate lung cancer in mice. Cancer Res 53, 1484-8.

Allard, W.J., Moran, C.A., Nagel, E., Collins, G. and Largen, M.T. (1992). Antigen binding properties of highly purified bispecific antibodies. Mol Immunol 29, 1219-27.

Allen, T.M. (1994) Long-circulating (sterically stabilized) liposomes for targeted drug delivery. Trends Pharmacol Sci 15, 215-20.

Allen, T.M. (1997) Liposomes: opportunities in drug delivery. Drugs 54, suppl. 4, 8-14.

Allen, T.M., Ahmad, I., Lopes de Menezes, D.E. and Moase, E.H. (1995) Immunoliposome-mediated targeting of anti-cancer drugs *in vivo*. Biochem Soc Trans 23, 1073-9.

Allen, T.M. and Chonn, A. (1987) Large unilamellar liposomes with low uptake into the reticuloendothelial system. FEBS Lett 223, 42-6.

Allen, T.M., Hansen, C., Martin, F., Redemann, C. and Yau-Young, A. (1991) Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives *in vivo*. Biochim Biophys Acta 1066, 29-36.

Allen, T. M., Mehra, T., Hansen, C. and Chin, Y. C. (1992) Stealth liposomes: an improved sustained release system for 1-beta-D-arabinofuranosylcytosine. Cancer Res 52, 2431-9.

Allen, T.M., and Moase, E.H. (1996) Therapeutic opportunities for targeted liposomal drug delivery. Adv Drug Deliv Rev 21, 117-33.

Alving, C.R. (1995) Lipopolysaccharide, lipid A and liposome containing lipid A as immunological adjuvants. Immunobiology 187, 430-46.

Anderson, P.M., Crist, W., Hasz, D., Carroll, A.J., Myers, D.E. and Uckun, F.M. (1992). G19.4 (alpha CD3) x B43(alpha CD19) monoclonal antibody heteroconjugate triggers CD19 antigen-specific lysis of t(4;11) acute lymphoblastic leukemia cells by activated CD3 antigen-positive cytotoxic T cells. Blood 80, 2826-34.

Anderson, P.M., Hansen, D.C., Hasz, D.E., Halet, M.R., Blazar, B.R. and Ochoa, A.C. (1994) Cytokines in liposomes: preliminary studies with IL-1, IL-2, IL-6, GM-CSF and IFN gamma. Cytokine 6, 92-101.

Aragnol D. and Leserman L.D. (1986) Immune clearance of liposomes inhibited by an anti-Fc receptor antibody *in vivo*. Proc Natl Acad Sci USA 83, 2699-703.

Auriol, J., Guesdon, J.L., Mazie, J.C. and Nato, F. (1994). Development of a bispecific monoclonal antibody for use in molecular hybridisation. J Immunol Methods 169, 123-33.

Azuma, A., Yagita, H., Matsuda, H., Okumura, K. and Niitani, H. (1992). Induction of intercellular adhesion molecule 1 on small cell lung carcinoma cell lines by gamma-interferon enhances spontaneous and bispecific anti-CD3 x antitumor antibody-directed lymphokine activated killer cell cytotoxicity. Cancer Res 52, 4890-4.

Bally, M. B., Masin, D., Nayar, R., Cullis, P. R. and Mayer, L. D. (1994) Transfer of liposomal drug carriers from the blood to the peritoneal cavity of normal and ascitic tumor-bearing mice. Cancer Chemother Pharmacol 34, 137-46.

Bakker-Woudenberg, I.A., Len-Kate, M.T., Stearne-Cullen, L.E. and Woodle, M.C. (1994) Efficacy of gentamicin or ceftazidime entrapped in liposomes with prolonged blood circulation and enhanced localization in Klebsiella pneumoniae infected lung tissue. J Infect Dis 171, 938-47.

Bangham, A.D., Standish, M.M. and Watkins, J.C. (1965) The action of steroids and streptolysin S on the permeability of phospholipid structures to cations. J Mol Biol 13, 138-44.

Barr, I.G., MacDonald, H.R., Buchegger, F. and von Fliedner, V. (1987). Lysis of tumor cells by the retargeting of murine cytolytic T lymphocytes with bispecific antibodies. Int J Cancer 40, 423-9.

Baumann, J.G.J. (1985) Fluorescence microscopical hybridocytochemistry. Acta Histochem 31, 9-18.

Baxter, L.T., Yuan, F. and Jain, R.K. (1992) Pharmacokinetic analysis of the perivascular distribution of bifunctional antibodies and haptens: comparison with experimental data. Cancer Res 52, 5838-44.

Bayer, E.A. and Wilchek, M. (1990) Avidin- and streptavidin-containing probes. Methods in Enzymology (M. Wilchek and E.A. Bayer, eds), vol. 184, pp174-185. Academic Press, San Diego.

Bayer, E.A., Ben-Hur, H. and Wilchek, M. (1986) A sensitive enzyme assay for biotin, avidin, and streptavidin. Anal Biochem 154, 367-70.

Berg, J., Lotscher, E., Steimer, K.S., Capon, D.J., Baenziger, J., Jack, H.M. and Wabl, M. (1991). Bispecific antibodies that mediate killing of cells infected with human immunodeficiency virus of any strain. Proc Natl Acad Sci USA 88, 4723-7.

Berger, M. (1979) Antibodies that bind biotin and inhibit biotin-containing enzymes. Methods Enzymol 62, 319-26.

Berkova, N., Karawajew, L., Korobko, V., Behrsing, O., Micheel, B., Shamborant, O., Stukatcheva, E. and Shingarova, L. (1996). Development of an enzyme immunoassay for the measurement of human tumour necrosis factor-alpha (hTNF-alpha) using bispecific antibodies to hTNF-alpha and horseradish peroxidase. Biotechnol Appl Biochem 23, 163-71.

Bloch, B. (1993) Biotinylated probes for in situ hybridization histochemistry: use for mRNA detection. J Histochem Cytochem 41, 1751-4.

Bolhuis, R.L., Lamers, C.H., Goey, S.H., Eggermont, A.M., Trimbos, J.B., Stoter, G., Lanzavecchia, A., di Re, E., Miotti, S. and Raspagliesi, F. (1992). Adoptive immunotherapy of ovarian carcinoma with bs-MAb-targeted lymphocytes: a multicenter study. Int J Cancer - Suppl 7, 78-81.

Bolhuis, R.L., Sturm, E. and Braakman, E. (1991). T cell targeting in cancer therapy. Cancer Immunol Immunother 34, 1-8.

Bonardi, M.A., Bell, A., French, R.R., Gromo, G., Hamblin, T., Modena, D., Tutt, A.L. and Glennie, M.J. (1992). Initial experience in treating human lymphoma with a combination of bispecific antibody and saporin. Int J Cancer - Suppl 7, 73-7.

Bonardi, M.A., French, R.R., Amlot, P., Gromo, G., Modena, D. and Glennie, M.J. (1993). Delivery of saporin to human B-cell lymphoma using bispecific antibody: targeting via CD22 but not CD19, CD37, or immunoglobulin results in efficient killing. Cancer Res 53, 3015-21.

Bos, R. and Nieuwenhuizen, W. (1992a). Enhanced transfection of a bacterial plasmid into hybridoma cells by electroporation: application for the selection of hybrid hybridoma (quadroma) cell lines. Hybridoma 11, 41-51.

Bos, R. and Nieuwenhuizen, W. (1992b). The potential improvement of thrombolytic therapy by targeting with bispecific monoclonal antibodies: why they are used and how they are made. Biotherapy 5, 187-99.

Branscomb, E.E., Runge, M.S., Savard, C.E., Adams, K.M., Matsueda, G.R. and Haber, E. (1990). Bispecific monoclonal antibodies produced by somatic cell fusion increase the potency of tissue plasminogen activator. Thromb Haemost 64, 260-6.

Brenner, M.B., Trowbridge, I.S. and Strominger, J.L. (1985). Cross-linking of human T cell receptor proteins: association between the T cell idiotype beta subunit and the T3 glycoprotein heavy subunit. Cell 40, 183-90.

Canevari, S., Menard, S., Mezzanzanica, D., Miotti, S., Pupa, S.M., Lanzavecchia, A. and Colnaghi, M.I. (1988). Anti-ovarian carcinoma anti-T3 heteroconjugates or hybrid antibodies induce tumor cell lysis by cytotoxic T-cells. Int J Cancer - Suppl 2, 18-21.

Canevari, S., Stoter, G., Arienti, F., Bolis, G., Colnaghi, M.I., Di Re, E.M., Eggermont, A.M., Goey, S.H., Gratama, J.W. and Lamers, C.H. (1995). Regression of advanced ovarian carcinoma by intraperitoneal treatment with autologous T lymphocytes retargeted by a bispecific monoclonal antibody. J Natl Cancer Inst 87, 1463-9.

Carucci, A.F. (1970) Methods Enzymol 18A, 379.

Chaiet, L. and Wolf, F.J. (1964) The properties of streptavidin, a biotin-binding protein produced by streptomycetes. Arch Biochem Biophys 106, 1-5.

Chamow, S.M., Zhang, D., Tan, X.Y., Mhatre, S.M., Marsters, S.A., Peers, D.H., Byrn, R.A., Ashkenazi, A. and Junghans, R.P. (1995). A humanized, bispecific immunoadhesin-antibody that retargets CD3+ effectors to kill HIV-1-infected cells. J Hematother 4, 439-46.

Chang, D.C. (1989). Cell poration and cell fusion using an oscillating electric field. Biophys J 56, 641-52.

Chang, Y.S., Wu, R.J., Chang, R.J. and Shiuan, D. (1994) Determination of biotin concentration by a competitive enzyme-linked immunosorbent assay (ELISA) method. J Biochem Biophys Methods 29, 321-29.

Charpie, J.R., Runge, M.S., Matsueda, G.R. and Haber, E. (1990). A bispecific antibody enhances the fibrinolytic potency of single-chain urokinase. Biochemistry 29, 6374-8.

Chatal, J.F., Faivre-Chauvet, A., Bardies, M., Peltier, P., Gautherot, E. and Barbet, J. (1995). Bifunctional antibodies for radioimmunotherapy. Hybridoma 14, 125-8.

Cheong, H.S., Chang, J.S., Park, J.M. and Byun, S.M. (1990). Affinity enhancement of bispecific antibody against two different epitopes in the same antigen. Biochem Biophys Res Commun 173, 795-800.

Chetanneau, A., Barbet, J., Peltier, P., Le Doussal, J.M., Gruaz-Guyon, A., Bernard, A.M., Resche, I., Rouvier, E., Bourguet, P. and Delaage, M. (1994). Pretargetted imaging of colorectal cancer recurrences using an 111In-labelled bivalent hapten and a bispecific antibody conjugate. Nucl Med Commun 15, 972-80.

Cook, A.G. and Wood, P.J. (1994). Chemical synthesis of bispecific monoclonal antibodies: potential advantages in immunoassay systems. J Immunol Methods 171, 227-37.

Cooper A. J. L. (1983) Biochemistry of sulfur-containing amino acid. Ann Rev Biochem 52, 187-222.

Cooper, M.L. (1996) Noninfectious gene transfer and expression systems for cancer genetherapy. Semin Oncol 23, 172-87.

Corvalan, J.R., Smith, W., Gore, V.A. and Brandon, D.R. (1987). Specific in vitro and *in vivo* drug localisation to tumour cells using a hybrid-hybrid monoclonal antibody recognising both carcinoembryonic antigen (CEA) and vinca alkaloids. Cancer Immunol Immunother 24, 133-7.

Cotton, R.G. and Milstein, C. (1973). Letter: Fusion of two immunoglobulin-producing myeloma cells. Nature 244, 42-3.

Dakshinamurti, K., Bhullar, R.P., Scoot, A., Rector, E.S., Delespesse, G. and Sehon, A.H. (1986) Production and characterization of a monoclonal antibody to biotin. Biochem J 237, 477-82.

Davico Bonino, L., De Monte, L.B., Spagnoli, G.C., Vola, R., Mariani, M., Barone, D., Moro, A.M., Riva, P., Nicotra, M.R. and Natali, P.G. (1995). Bispecific monoclonal antibody anti-CD3 x anti-tenascin: an immunotherapeutic agent for human glioma [published erratum appears in Int J Cancer 1995 Jul 28;62(3):364]. Int J Cancer 61, 509-15.

Debs, R.J., Heath, T.D. and Papahajopoulos, D. (1987) Targeting of anti-Thy 1.1 monoclonal antibody conjugated liposomes in Thy 1.1 mice after intravenous administration. Biochim Biophys Acta 901, 183-90.

DeGast, G.C., Van Houten, A.A., Haagen, I.A., Klein, S., De Weger, R.A., Van Dijk, A., Phillips, J., Clark, M. and Bast, B.J. (1995). Clinical experience with CD3 x CD19 bispecific antibodies in patients with B cell malignancies. J Hematother 4, 433-7.

DeKruif, J. and Logtenberg, T. (1996). Leucine zipper dimerized bivalent and bispecific scFv antibodies from a semi-synthetic antibody phage display library. J Biol Chem 271, 7630-4.

DeLau, W.B., Boom, S.E., Heije, K., Griffioen, A.W., Braakman, E., Bolhuis, R.L., Tax, W.J., Clevers, H. and Bast, B.J. (1992). Heterodimeric complex formation with CD8 and TCR by bispecific antibody sustains paracrine IL-2-dependent growth of CD3+ CD8+ T cells. J Immunol 149, 1840-6.

DeLau, W.B., Van Loon, A.E., Heije, K., Valerio, D. and Bast, B.J. (1989). Production of hybrid hybridomas based on HAT(s)-neomycin(r) double mutants. J Immunol Methods 117, 1-8.

Della-Penna, D., Christofferson, R.E. and Bennett, A.B. (1986) Biotinylated proteins as molecular weight standards on western blots. Anal Biochem 152, 329-32.

Demanet, C., Brissinck, J., De Jonge, J. and Thielemans, K. (1996). Bispecific antibody-mediated immunotherapy of the BCL1 lymphoma: increased efficacy with multiple injections and CD28-induced costimulation. Blood 87, 4390-8.

DeMarie, S. (1996) Liposomal and lipid based formulations of Amphotericin B. Leukaemia 10, Suppl 2, 93-6.

Demonte, L.B., Dellabona, P., Nistico, P., Tecce, R., Camagna, M., Reyna, A., Momo, M., Natali, P.G., Mariani, M. and Malavasi, F. (1991). Retrovirus-derived shuttle vectors in the generation of murine bispecific MoAbs to be used in ex vivo and *in vivo* immunotherapy. Annali dell Istituto Superiore di Sanita 27, 133-7.

Deramoudt, E.X., Gilard, C., Lepine, N., Alonso, J.M. and Romet-Lemonne, J.L. (1992). Bispecific anti-human red blood Rhesus-D antigen x anti Fc gamma RI targeted antibody-dependent cell-mediated cytotoxicity and phagocytosis by mononuclear leucocytes. Clin Exp Immunol 89, 310-4.

De Sutter, K. and Fiers, W. (1994). A bifunctional murine::human chimeric antibody with one antigen-binding arm replaced by bacterial beta-lactamase. Mol Immunol 31, 261-7.

Diamandis, E.P. and Christopoulos, T.K. (1991) The biotin-(strept)avidin system: principles applications in biotechnology. Clin Chem 37, 625-36.

Dillehay, L.E., Mayer, R., Zhang, Y.G., Shao, Y., Song, S.Y., Mackensen, D.G. and Williams, J.R. (1995). Prediction of tumor response to experimental radioimmunotherapy with 90Y in nude mice. Int J Radiat Oncol Biol Phys 33, 417-27.

Ding, L., Samuel, J., MacLean, G.D., Noujaim, A.A., Diener, E. and Longenecker, B.M. (1990) Effective drug-antibody targeting using monoclonal antibody against the proliferative compartment of mammalian squamous carcinoma. Cancer Immunol Immunother 31, 105-9.

Ehrlich, P. (1906) The relations existing between chemical constitution, distribution and pharmacological action. Collected studies on immunity (P. Ehrlich, Ed.), pp404-443, John Wiley and Sons, Ltd., New York.

Espinola, I. R., Dunnick, J. K., Goris, M. L. and Kriss, J. P. (1979) Radiolabeled liposomes as metabolic and scanning tracers in mice. II. In-III oxime compared with Tc-99m-DTPA, entrapped in multilamellar lipid vesicles. J Nucl Med 20, 434-40.

Fanger, M.W., Ball, E.D. and Drakeman, D.L. (1995). Comments on the Fourth International Conference on Bispecific Antibodies and Cellular Cytotoxicity, Duck Key, Florida, March 1-5, 1995 [editorial]. J Hematother 4, 345-9.

Ferrini, S., Prigione, I., Mammoliti, S., Colnaghi, M.I. Menard, S., Moretta, A. and Moretta, L. (1989). Retargeting of T-cell receptor gamma/delta+ lymphocytes against tumor cells by bispecific monoclonal antibodies. Int J Cancer - Suppl 4, 53-5.

Fiske, C.H. and Subbarow, Y. (1925) The colorimetric determination of phosphorus. J Biol Chem 66, 375-400.

Forster, A.C., McInnes, J.L., Skingle, D.C. and Symons, R.H. (1985) Non-radioactive hybridization probes prepared by the chemical labeling of DNA and RNA with a novel reagent, photobiotin. Nucleic Acids Res 13, 745-61.

French, R.R., Bell, A.J., Hamblin, T.J., Tutt, A.L. and Glennie, M.J. (1996). Response of B-cell lymphoma to a combination of bispecific antibodies and saporin. Leuk Res 20, 607-17.

French, R.R., Hamblin, T.J., Bell, A.J., Tutt, A.L. and Glennie, M.J. (1995). Treatment of B-cell lymphomas with combination of bispecific antibodies and saporin. Lancet 346, 223-4.

Friguet, B., Chaffotte, A.F., Djavadi-Ohaniance, L. and Goldberg, M.E. (1985) Measurements of the true affinity constant in solution of antigen-antibody complexes by enzyme-linked immunosorbent assay. J Immunol Methods 77, 305-19.

Gabizon, A.A. (1992) Selective tumour localization and improved therapeutic index of anthracyclines encapsulated in long circulating liposomes. Cancer Res 52, 891-6.

Gabizon, A.A., Chemla, M., Tzemach, D., Horowitz, A. T., and Goren, D. (1996) Liposome longevity and stability in circulation: effects on the *in vivo* delivery to tumors and therapeutic efficacy of encapsulated anthracyclines. J Drug Target 3, 391-8.

Gabizon, A.A., Isaacson, R. and Libson, E. (1994) Clinical studies of liposome encapsulated doxorubicin. Acta Oncol 33 779-86.

Gabizon, A.A., Meshorer, A. and Barenholz, Y. (1986) Comparative long-term study of the toxicities of free and liposome-associated doxorubicin in mice after intravenous administration. J Natl Cancer Inst 77, 459-69.

Gabizon, A.A. and Paphadjopoules, D. (1988) Liposomal formulation with prolonged circulation time in blood and enhanced uptake in tumors. Proc Natl Acad Sci USA 85, 6949-53.

Gabizon, A., Price, D. C., Huberty, R. S. and Papahadjopoulos, D. (1990) Effect of liposome composition and other factors on the targeting of liposomes to experimental tumors: biodistribution and imaging studies. Cancer Res 50, 6371-8.

Gallagher, S.R. and Smith, J.A. (1991) One dimensional gel electrophoresis. in Current protocols in immunology, vol II, John Wiley and Sons, Inc., New York, N.Y., pp 8.4.1-8.4.21.

Glassy, M. (1998) Creating hybridomas by electrofusion. Nature 333, 579-80.

Glennie, M.J., McBride, H.M., Worth, A.T. and Stevenson, G.T. (1987). Preparation and performance of bispecific F(ab' gamma)2 antibody containing thioether-linked Fab' gamma fragments. J Immunol 139, 2367-75.

Goins, B., Klipper, R., Rudolph, A. S., Cliff, R. O., Blumhardt, R. and Phillips, W. T. (1993) Biodistribution and imaging studies of technetium-99m-labeled liposomes in rats with focal infection. J Nucl Med 34, 2160-8.

Goodwin, D.A. (1987). Pharmacokinetics and antibodies. J Nucl Med 28, 1358-62.

Goodwin, D.A., Meares, C.F., Watanabe, N., McTigue, M., Chaovapong, W., Ransone, C.M., Renn, O., Greiner, D.P., Kukis, D.L. and Kronenberger, S.I. (1994). Pharmacokinetics of pretargeted monoclonal antibody 2D12.5 and 88Y-Janus-2-(p-nitrobenzyl)-1,4,7,10-tetraazacyclododecanetetraaceti c acid (DOTA) in BALB/c mice with KHJJ mouse adenocarcinoma: a model for 90Y radioimmunotherapy. Cancer Res 54, 5937-46.

Green, N.M. (1963) Avidin. 1. The use of ^{14}C-biotin for kinetic studies and for assay. Biochem J 89, 585-91.

Green, N.M. (1965) A spectrophotometric assay for avidin and biotin based on binding of dyes by avidin. Biochem J 94, 23-4.

Green, N.M. (1970) Spectrophotometric determination of avidin and biotin. In: J.L. Langone and H.V. Vanukis (Eds.), Methods Enzymol 74, Academic Press, NY, pp418-24.

Gregoriadis, G. (1973) Drug entrapment in liposomes. FEBS Lett 36, 292-6.

Gregoriadis, G. and Florence, A. T. (1993) Liposomes in drug delivery. Drugs 45, 15-28.

Gregoriadis, G., Neerunjun, D.E. and Hunt, R. (1977) Attempts to improve localization in tumor tissues. Life Sci 21, 357-70.

Gruber, M., Schodin, B.A., Wilson, E.R. and Kranz, D.M. (1994). Efficient tumor cell lysis mediated by a bispecific single chain antibody expressed in Escherichia coli. J Immunol 152, 5368-74.

Haagen, I.A., van de Griend, R., Clark, M., Geerars, A., Bast, B. and de Gast, B. (1992). Killing of human leukaemia/lymphoma B cells by activated cytotoxic T lymphocytes in the presence of a bispecific monoclonal antibody (alpha CD3/alpha CD19). Clin Exp Immunol 90, 368-75.

Hakalahti, L., Vihko, P., Henttu, P., Autio-Harmainen, H., Soini, Y. and Vihko, R. (1993). Evaluation of PAP and PSA gene expression in prostatic hyperplasia and prostatic carcinoma using northern-blot analyses, in situ hybridization and immunohistochemical stainings with monoclonal and bispecific antibodies. Int J Cancer 55, 590-7.

Harlow, E.D. and Lane, D. (1988) Storing and purifying antibodies. Antibodies: A laboratory manual. (E.D. Harlow, and D. Lane, Eds.), pp298, John Wiley and Sons, Ltd., New York.

Hayden, M.S., Gilliland, L.K. and Ledbetter, J.A. (1997) Antibody engineering. Current Opinion in Immunology 9: 201-12.

Heath, T.D., Bragman, K.S., Matthay, K.K., Lopez-Straubinger, N.G. and Papahadjopoulos, D. (1984) Antibody-directed liposomes: the development of a cell-specific cytotoxic agent. Biochem Soc Trans 12, 340-2.

Heath, T.D., Montgomery, J.A., Piper, J.R. and Papahadjopoulos, D. (1983) Antibody-targeted liposomes: increased in specific toxicity of methotrexate-g-aspartate. Proc Natl Acad Sci USA 80, 1377-81.

Hermansen, G.T. (1996) Bioconjugate techniques, Academic Press, NY, USA.

Hernandez M. and Rosenthall, L. (1980) A cross-over study comparing the kinetics of Tc-99m-labeled isopropyl and p-butyl IDA analogs in patients. Clin Nucl Med 5, 159-65.

Holliger, P., Prospero, T. and Winter, G. (1993). "Diabodies": small bivalent and bispecific antibody fragments. Proc Natl Acad Sci USA 90, 6444-8.

Hopman, A.H.N., Wiegant, J. and Van Duijn, P (1986) A new hybridocytochemical method based on mercurated nucleic acid probes and sulfhydril-hapten ligands. Histochemistry 84, 169-78.

Hsieh-Ma, S.T., Eaton, A.M., Shi, T. and Ring, D.B. (1992). In vitro cytotoxic targeting by human mononuclear cells and bispecific antibody 2B1, recognizing c-erbB-2 protooncogene product and Fc gamma receptor III. Cancer Res 52, 6832-9.

Huang, S.K., Lee, K.D., Hong, K., Friend, D.S. and Papahadjopoulos, D. (1992) Microscope localization of sterically stabilized liposomes in colon carcinoma bearing mice. Cancer Res 52, 5135-43.

Huang, S.K., Martin, F.J., Jay, G., Vogel, J. Papahadjopoulos, D. and Friend, D.S. (1993) Extravasation and transcytosis of liposomes in kaposi's sarcoma-like dermal lesions of transgenic mice bearing the HIV tat gene. Am J Pathol 143, 10-4.

Imura, Y., Stassen, J.M., Kurokawa, T., Iwasa, S., Lijnen, H.R. and Collen, D. (1992). Thrombolytic and pharmacokinetic properties of an immunoconjugate of single-chain urokinase-type plasminogen activator (u-PA) and a bispecific monoclonal antibody against fibrin and against u-PA in baboons. Blood 79, 2322-9.

Jantscheff, P., Winkler, L., Karawajew, L., Kaiser, G., Bottger, V. and Micheel, B. (1993). Hybrid hybridomas producing bispecific antibodies to CEA and peroxidase isolated by a combination of HAT medium selection and fluorescence activated cell sorting. J Immunol Methods 163, 91-7.

Jones, M.A., Kilpatrick, P.K. and Carbonell, R.G. (1993) Preparation and characterization of bifunctional unilamellar vesicles for enhanced immunosorbent assay. Biotechnol Prog 9, 242-58.

Jones, M.A., Kilpatrick, P.K. and Carbonell, R.G. (1994) Competitive immunosorbent assays for biotin using bifunctional unilamellar vesicles. Biotechnol Prog 10, 174-86.

Jones, M.A., Kilpatrick, P.K. and Carbonell, R.G. (1996) Competitive immunosorbent assays using ligand-enzyme conjugates and bifunctional liposomes: theory and experiment. Biotechnol Prog 12, 519-26.

Jung, G., Freimann, U., Von Marschall, Z., Reisfeld, R.A. and Wilmanns, W. (1991). Target cell-induced T cell activation with bi- and trispecific antibody fragments. Eur J Immunol 21, 2431-5.

Kaneko, T., Fusauchi, Y., Kakui, Y., Masuda, M., Akahoshi, M., Teramura, M., Motoji, T., Okumura, K., Mizoguchi, H. and Oshimi, K. (1993). A bispecific antibody enhances cytokine-induced killer-mediated cytolysis of autologous acute myeloid leukemia cells. Blood 81, 1333-41.

Karawajew, L., Behrsing, O., Kaiser, G. and Micheel, B. (1988). Production and ELISA application of bispecific monoclonal antibodies against fluorescein isothiocyanate (FITC) and horseradish peroxidase (HRP). J Immunol Methods 111, 95-9.

Karawajew, L., Micheel, B., Behrsing, O. and Gaestel, M. (1987). Bispecific antibody-producing hybrid hybridomas selected by a fluorescence activated cell sorter. J Immunol Methods 96, 265-70.

Karlsson, R., Michaelsson, A. and Mattsson, L. (1991) Kinetic analysis of monoclonal antibody-antigen interactions with a new biosensor based analytical system. J Immunol Methods 145, 229-40.

Kim, S. (1994) Liposomes as carriers for cancer chemotherapy: current status and future prospects. Drugs 46, 618-38.

Klingersmith, W. C., Fritzberg, A. R. and Spitzer, V. M. (1981) Clinical comparison of Tc-99m diisopropyl-IDA and diethyl-IDA for evaluation of the hepatobiliary system. Radiology 140, 791-5.

Knuth, A., Bernhard, H., Jager, E., Wolfel, T., Karbach, J., Jaggle, C., Strittmatter, W. and Meyer zum Buschenfelde, K.H. (1994). Induction of tumour cell lysis by a bispecific antibody recognising epidermal growth factor receptor (EGFR) and CD3. Eur J Cancer 30A, 1103-7.

Kohler, G. and Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256, 495-7.

Kontsekova, E., Novak, M. and Kontsek, P. (1992). [Bispecific antibodies: present status and possibile applications]. Bratislavske Lekarske Listy, 93, 405-10.

Koolwijk, P., Rozemuller, E., Stad, R.K., De Lau, W.B. and Bast, B.J. (1988). Enrichment and selection of hybrid hybridomas by Percoll density gradient centrifugation and fluorescent-activated cell sorting. Hybridoma 7, 217-25.

Kostelny, S.A., Cole, M.S. and Tso, J.Y. (1992). Formation of a bispecific antibody by the use of leucine zippers. J Immunol 148, 1547-53.

Kranenborg, M.H., Boerman, O.C., Oosterwijk-Wakka, J.C., de Weijert, M.C., Corstens, F.H. and Oosterwijk, E. (1995). Development and characterization of anti-renal cell carcinoma x antichelate bispecific monoclonal antibodies for two-phase targeting of renal cell carcinoma. Cancer Res 55, 5864s-7s.

Kranz, D.M., Gruber, M. and Wilson, E.R. (1995). Properties of bispecific single chain antibodies expressed in Escherichia coli. J Hematother 4, 403-8.

Kreutz, F.T. and Suresh, M.R. (1997). Novel bispecific immunoprobe for rapid and sensitive detection of prostate-specific antigen. Clin Chem 43, 649-56.

Kreutz, F.T. and Suresh, M.R. (1995). Bispecific monoclonal anti-CA125 X anti-peroxidase antibodies in the measurement of the ovarian carcinoma antigen. J Tumor Marker Oncol 10, 45-53.

Kreutz, F.T., Wishart, D.S. and Suresh, M.R. (1998) An efficient bispecific monoclonal antibody purification using gradient thiophilic affinity chromatography. J Chromatogr B Biomed Sci Appl (in press).

Kubo, A., Nakamura, K. and Sammiya, T. (1993) Indium-111-labeled liposomes: dosimetry and tumor detection in patients with cancer. Eur J Nucl Med 20, 107-13.

Kung, V.T., Maxim, P.E., Veltri, R.W. and Martin, F.J. (1985) Antibody-bearing liposomes improve agglutination of latex particles used in clinical diagnostic assays. Biochim Biophys Acta 893, 105-9.

Kurokawa, T., Iwasa, S., Kakinuma, A., Stassen, J.M., Lijnen, H.R. and Collen, D. (1991). Enhancement of clot lysis in vitro and *in vivo* with a bispecific monoclonal antibody directed against human fibrin and against urokinase-type plasminogen activator. Thromb Haemost, 66, 684-93.

Laemmli, U.K. (1970) Cleavage of structural protein during the assembly of the head of bacteriophage T4. Nature 227, 680-5.

Lanzavecchia, A. and Scheidegger, D. (1987). The use of hybrid hybridomas to target human cytotoxic T lymphocytes. Eur J Immunol 17, 105-11.

Lasic, D. (1992) Liposomes. Am Sci 80: 20-31.

Leong, M.M., Milstein, C. and Pannell, R. (1986) Luminescent detection method for immunodot, western, and Southern blots. J Histochem Cytochem 34, 1645-50.

Leserman, L. and Machy, P. (1987) Ligand targeting of liposomes. Liposome: from biophysics to therapeutics. (M.J. Ostro, ed) pp157-194. Dekker, NY. USA.

Li, C.K.N. (1985) ELISA-based determination of immunological binding constants. Mol Immunol 22, 321-7.

Li, W.P., Zuber, C. and Roth, J. (1993) Use of phaseolus vulgaris leukoagglutinating lectin in histochemical and blotting techniques: a comparison of digoxigenin and biotin labeled lectins. Histochemistry 100, 347-56.

Lim, H. J., Masin, D., Madden, T. D. and Bally, M. B. (1997) Influence of drug release characteristics on the therapeutic activity of liposomal mitoxantrone. J Pharmacol Exp Ther 281, 566-73.

Liu, L., Barth, R.F., Adams, D.M., Soloway, A.H. and Reisfeld, R.A. (1995). Bispecific antibodies as targeting agents for boron neutron capture therapy of brain tumors. J Hematother 4, 477-83.

Liu, M.A., Kranz, D.M., Kurnick, J.T., Boyle, L.A., Levy, R. and Eisen, H.N. (1985). Heteroantibody duplexes target cells for lysis by cytotoxic T lymphocytes. Proc Natl Acad Sci USA 82, 8648-52.

Loberg M.D., Cooper, M. and Harvey, E. (1976) Development of new radiopharmaceuticals based on N-substitution of iminodiacetic acid. J Nucl Med 17, 633-8.

Longenecker, B.M., Willians, D.J., Maclean, G.D., Selvaraj, S., Suresh, M.R. and Noujaim, A.A. (1987) Monoclonal antibodies and synthetic tumor associated glycoconjugates in the study of the expression of Thomsen-Friedenreich-like and Tn like antigens on human cancers. J. Natl. Cancer Inst. 78, 489.

Longman, S.A., Cullis, P.R., Choi, L., de Jong, G. and Bally, M.B. (1995) A two-step targeting approach for delivery of doxorubicin-loaded liposomes to tumour cells *in vivo*. Cancer Chemother Pharmacol 36, 91-101.

Loughrey, H.C., Choi, L.S., Wong, K.F., Cullis, P.R. and Bally, M.B. (1993a) Preparation of streptavidin-liposomes for use in ligand-specific targeting applications. Liposome technology, vol 3 (G. Gregoriadis, Ed.) pp163-178, CRC, Boca Raton, Florida.

Loughrey, H. C., Ferraretto, A., Cannon, A. M., Acerbis, G., Sudati, F., Bottiroli, G., Masserini, M. and Soria, M. R. (1993b) Characterization of biotinylated liposomes for *in vivo* targeting applications. FEBS Lett 332, 183-88.

Loughrey, H.C., Wong, K.F., Choi, L.S., Cullis, P.R. and Bally, M.B. (1990) Protein-liposome conjugates with defined size distributions. Biochim Biophys Acta 1028, 73-81.

Love, W. G., Amos, N., Williams, B. D. and Kellaway, I. W. (1989) Effect of liposome surface charge on the stability of technetium (99mTc) radiolabelled liposomes. J Microencapsul 6, 105-13.

Mallender, W.D., Ferreira, S.T., Voss, E.W., Jr. and Coelho-Sampaio, T. (1994). Inter-active-site distance and solution dynamics of a bivalent-bispecific single-chain antibody molecule. Biochemistry 33, 10100-8.

Maruyama, K., Kennel, S. and Huang, L. (1990) Lipid composition is important for highly efficient target binding and retention of immunoliposomes. Proc Natl Acad Sci USA 87, 5744-8.

Matthay, K.K., Heath, T.D. and Papahadjopoulos, D. (1984) Specific enhancement of drug delivery to AKR lymphoma by antibody-targeted small unilamellar vesicles. Cancer Res 44, 1880-6.

Mayer, L. D., Nayar, R., Thies, R. L., Boman, N. L., Cullis, P. R. and Bally, M. B. (1993) Identification of vesicle properties that enhance the antitumour activity of liposomal vincristine against murine L1210 leukemia. Cancer Chemother Pharmacol 33, 17-24.

Mayer, L. D. and St.-Onge, G. (1995) Determination of free and liposome-associated doxorubicin and vincristine levels in plasma under equilibrium conditions employing ultrafiltration techniques. Anal Biochem 232, 149-57.

Mayhew, E.G., Lasic, D., Babbar, S. and Martin, F.J. (1992) Pharmacokinetics and antitumor activity of epirubicin encapsulated in long-circulation liposomes incorporating a polyethylene glycol-derivatized phospholipid. Int J Cancer 51, 302-9.

McDougall, I. R., Dunnick, J. K., Goris, M. L. and Kriss, J. P. (1975) *In vivo* distribution of vesicles loaded with radiopharmaceuticals: a study of different routes of administration. J Nucl Med 16, 488-92.

McGuinness B.T., Walter G., Fitzgerald K., Schuler P., Mahoney, W., Duncan A.R. and Hoogenboom H.R. (1996) Phage diabody repertoires for selection of large numbers of bispecific antibody fragments. Natl Biotechnol 14, 1149-53.

Milstein, C. and Cuello, A.C. (1983). Hybrid hybridomas and their use in immunohistochemistry. Nature 305, 537-40.

Mori, A., Kennel, S.J., van Borssum Waalkes, M., Scherphof, G.L. and Huang, L. (1995) Characterization of organ-specific immunoliposomes for delivery of 3',5'-O-dipalmitoyl-5-fluoro-2'-deoxyuridine in a mouse lung-metastasis model. Cancer Chemother Pharmacol 35, 447-56.

Mori, A., Klibanov, A.L., Torchilin, V.P. and Huang, L. (1991) Influence of steric barrier activity of amphipathic poly(ethyleneglycol) and ganglioside GM1 on the circulation time of liposomes and on the target binding of immunoliposomes *in vivo*. FEBS Lett 284, 263-6.

Mori, A., Wu, S.P., Han, I., Khakhar, A.R., Perez-Soler, R. and Huang, L. (1996) *In vivo* activity of cis-bis-neodecanoato-trans-R,R-1,2 diaminocyclohexane platinum (II) formulated in long circulating liposomes. Cancer Chemother Pharmacol 37, 435-44.

New, R.R.C. (1990) Paper chromatography of labeled liposomes. Liposomes: Practical approach (R.R.C. New, Ed) pp105-162, I.R.L. Press, Oxford.

Nieto, A., Gaya, A., Jansa, M., Moreno, C. and Vives, J. (1984) Direct measurement of antibody affinity distribution by hapten-inhibition enzyme immunoassay. Mol Immunol 21, 537-43.

Nisonoff, A. and Rivers, M.M. (1961). Recombination of a mixture of univalent antibody fragments of different specificity. Arch Biochem Biophys 93, 460-2.

Nolan, O. and Kennedy O.R. (1990). Bifunctional antibodies: concept, production and applications. Biochim Biophys Acta 1040, 1-11.

Ogihara-Umeda, I., Sasaki, T., Toyama, H., Oda, K., Senda, M. and Nishigori, H. (1994) Rapid tumor imaging by active background reduction using biotin-bearing liposomes and avidin. Cancer Res 54, 463-7.

Ogihara-Umeda, I., Sasaki, T., Kojima, S. and Nishigori, H. (1996) Optimal radiolabeled liposomes for tumor imaging. J Nucl Med 37, 326-32.

Ohta, S., Tsukamoto, H., Watanabe, K., Makino, K., Kuge, S., Hanai, N., Habu, S. and Nishimura, T. (1995). Tumor-associated glycoantigen, sialyl Lewis(a) as a target for bispecific antibody-directed adoptive tumor immunotherapy. Immunol Lett 44, 35-40.

Oku, N., Namba, Y., Takeda, A. and Okada, S. (1993) Tumor imaging with technetium-99m-DTPA encapsulated in RES-avoiding liposomes. Nucl Med Biol 20, 407-12.

Pak, R.H., Primus, F.J., Rickard-Dickson, K.J., Ng, L.L., Kane, R.R. and Hawthorne, M.F. (1995). Preparation and properties of nido-carborane-specific monoclonal antibodies for potential use in boron neutron capture therapy for cancer. Proc Natl Acad of USA 92, 6986-90.

Papahadjopoulos, D., Allen, T.M., Gabizon, A., Mayhew, E., Matthay, K., Huang, S.K., Lee, K.D., Woodle, M.C., Lasic, D.D., Redemann, C. and Martin, F.J. (1991) Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy. Proc Natl Acad Sci USA 88, 11460-4.

Park, J.W., Hong, K. and Carter, P. (1995) Development of anti-p185HER2 immunoliposomes for cancer therapy. Proc Natl Acad Sci USA 92, 1327-31.

Phillips, W. T., Rudolph, A. S., Goins, B., Timmons, J. H., Klipper, R. and Blumhardt, R. (1992) A simple method for producing a technetium-99m-labeled liposome which is stable *in vivo*. Nucl Med Biol 19, 539-47.

Piran, U., Silbert-Shostek, D. and Barlow, E.H. (1993). Role of antibody valency in hapten-heterologous immunoassays. Clin Chem 39, 879-83.

Plant, A.L., Brizgys, M.V., Locasio-Brown, L. and Durst, R. (1989) A generic liposome reagent for immunoassays. Anal Biochem 176, 420-6.

Presant, C. A., Proffitt, R. T. and Turner, A. F. (1988) Successful imaging of human cancer with Indium-III labeled phospholipid vesicles. Cancer 62, 905-11.

Presant, C. A., Blayney, D., Proffitt, R. T., Turner, A. F., Williams, L. E., Nadel, H. I., Kennedy, P., Wiseman, C., Gala, K. and Crossley, R. J. (1990) Preliminary report: imaging of Kaposi sarcoma and lymphoma in AIDS with indium-111-labelled liposomes. Lancet 335, 1307-9.

Rahman, A., Treat, J., Roe, J. K., Potkul, L. A. and Alvord, W.G. (1990) Phase I clinical trial and pharmacokinetic evaluation of liposomal encapsulated doxorubicin. J Clin Oncol 8, 1093-100.

Rammensee, H.G., Julius, M.H., Nemazee, D., Langhorne, J., Lamers, R. and Kohler, G. (1987). Targeting cytotoxic T cells to antigen-specific B lymphocytes. Eur J Immunol 17, 433-6.

Ranson, M., Howell, A., Cheeseman, S. and Margison, J. (1996) Liposomal drug delivery. Cancer Treat Rev 22, 365-79.

Raso, V. and Griffin, T. (1981) Hybrid antibodies with dual specificity for the delivery of ricin to immunoglobulin-bearing target cells. Cancer Res 41, 2073.

Reading, C.J., Bator, J. and O'Kennedy, R. (1989) Bispecific antibodies. Int. J. Biotech. 6, 1.

Reddy, V.S. and Ford, C.H. (1993). Production of hybrids secreting bispecific antibodies recognising CEA and doxorubicin. Anticancer Res 13, 2077-83.

Rheinnecker M, Hardt C, Iiag L.L., Kufer P, Gruber R., Hoess A, Lupas A., Rottenberger C, Pluckthun A. and Pack P (1996) Multivalent antibody fragments with high functional affinity for a tumor associated carbohydrate antigen. J Immunol 157, 2989-97.

Ribeiro-da-Silva, A., Pioro, E.P. and Cuello, A.C. (1991). Substance P- and enkephalin-like immunoreactivities are colocalized in certain neurons of the substantia gelatinosa of the rat spinal cord: an ultrastructural double-labeling study. J Neurosci 11, 1068-80.

Richardson, V.J., Jeyasingh, K., Jewkes, R.F., Ryman, B.E. and Tattersall, M.H.N. (1978) Possible tumor localization of Tc-99m-labeled liposomes: effects of lipid composition, charge and liposome size. J Nucl Med 19, 1049-54.

Ridgway, J.B., Presta, L.G. and Carter, P. (1996). 'Knobs-into-holes' engineering of antibody CH3 domains for heavy chain heterodimerization. Protein Eng 9, 617-21.

Riley, L.K. and Caffrey, C.J. (1990) Identification of enterotoxigenic Esherichia coli by colony hybridization with nonradioactive digoxigenin-labelled DNA probes. J Clin Microbiol 28, 1465-8.

Roosnek, E. and Lanzavecchia, A. (1989). Triggering T cells by otherwise inert hybrid anti-CD3/antitumor antibodies requires encounter with the specific target cell. J Exp Med 170, 297-302.

Roosnek, E., Tunnacliffe, A. and Lanzavecchia, A. (1990). T cell activation by a bispecific anti-CD3/anti-major histocompatibility complex class I antibody. Eur J Immunol 20, 1393-6.

Rylatt, D.B., Kemp, B.E., Bundesen, P.G., John, M.A., EJ, O.R., Cottis, L.E., Miles, S.J., Khan, J.M., Dinh, D.P. and Stapleton, D. (1990). A rapid whole-blood immunoassay system. Med J Aust 152, 75-7.

Saha, G. B. (1987) Normal biodistribution of diagnostic radiopharmaceuticals, in Hladik, W. B., Saha, G. B. and Study, K. T. (eds) Essentials of nuclear medicine science, Williams and Wilkins, Baltimore, MD, pp3-19.

Sahin, U., Hartmann, F., Senter, P., Pohl, C., Engert, A., Diehl, V. and Pfreundschuh, M. (1990). Specific activation of the prodrug mitomycin phosphate by a bispecific anti-CD30/anti-alkaline phosphatase monoclonal antibody. Cancer Res 50, 6944-8.

Samuel J., Noujaim, A.A., Willans D.J., Brzezinska G.S., Haines, D.M. and Longenecker, B.M. (1989) A novel marker for basal (stem) cells of mammalian stratified squamous epithelia and squamous cell carcinomas. Cancer Res 49, 2465-70.

Schmidt, M., Hynes, N.E., Groner, B. and Wels, W. (1996). A bivalent single-chain antibody-toxin specific for ErbB-2 and the EGF receptor. Int J Cancer 65, 538-46.

Schmitt, J.J. Zimmermann, U. and Neil, G.A. (1989) Efficient generation of stable antibody forming hybridoma cells by electrofusion. Hybridoma 8, 107-14.

Schray K.J., Artz, P.G. and Hevey, R.C. (1988) Determination of avidin and biotin by fluorescence polarization. Anal Biochem 60, 853-5.

Schuhmacher, J., Klivenyi, G., Matys, R., Stadler, M., Regiert, T., Hauser, H., Doll, J., Maier-Borst, W. and Zoller, M. (1995). Multistep tumor targeting in nude mice using bispecific antibodies and a gallium chelate suitable for immunoscintigraphy with positron emission tomography. Cancer Res 55, 115-23.

Segal, D.M., Qian, J.H., Garrido, M.A., Perez, P., Winkler, D.F., Wunderlich, J.R., Snider, D.P., Valdayo, M.J. and Titus, J.A. (1988). Targeting of cytotoxic cells against tumors with heterocrosslinked, bispecific antibodies. Princess Takamatsu Symposia, 19, 323-31.

Senior, J. (1987) Fate and behavior of liposomes *in vivo*: a review of controlling factors. CRC Crit Rev Ther Drug Carrier Syst 3, 123-93

Senior, J., Delgado, C., Tilcock, C. and Gregoriadis, G. (1991) Influence of surface hydrophilicity of liposomes on their interaction with plasma protein and clearance from the circulation: studies with poly(ethylene glycol)-coated vesicles. Biochim Biophys Acta 1062, 77-82.

Shalaby, M.R., Shepard, H.M., Presta, L., Rodrigues, M.L., Beverley, P.C., Feldmann, M. and Carter, P. (1992). Development of humanized bispecific antibodies reactive with cytotoxic lymphocytes and tumor cells overexpressing the HER2 protooncogene. J Exp Med 175, 217-25.

Shroyer, K.P. and Nakane, P.K.J. (1983) Use of DNP-labeled cDNA for in situ hybridization. J Cell Biol 97, 377a

Skeggs HR. (1966) Microbiological assay of vitamin B12. Methods Biochem Anal 14, 53-62.

Smith, W., Jarrett, A.L., Beattie, R.E. and Corvalan, J.R. (1992). Immunoglobulins secreted by a hybrid-hybridoma: analysis of chain assemblies. Hybridoma, 11, 87-98.

Somasundaram, C., Arch, R., Matzku, S. and Zoller, M. (1996). Development of a bispecific F(ab')2 conjugate against the complement receptor CR3 of macrophages and a variant CD44 antigen of rat pancreatic adenocarcinoma for redirecting macrophage-mediated tumor cytotoxicity. Cancer Immunol Immunother 42, 343-50.

Songsivilai, S., Clissold, P.M. and Lachmann, P.J. (1989). A novel strategy for producing chimeric bispecific antibodies by gene transfection. Biochem Biophys Res Commun 164, 271-6.

Songsivilai, S. and Lachmann, P.J. (1990). Bispecific antibody: a tool for diagnosis and treatment of disease. Clin Exp Immunol 79, 315-21.

Spitz, M., Spitz, L., Thorpe, R. and Eugui, E. (1984) Intrasplenic primary immunization for the production of monoclonal antibodies. J Immunol Methods 70, 39-43.

Staerz, U.D. and Bevan, M.J. (1985). Cytotoxic T lymphocyte-mediated lysis via the Fc receptor of target cells. Eur J Immunol 15, 1172-7.

Staerz, U.D. and Bevan, M.J. (1986). Hybrid hybridoma producing a bispecific monoclonal antibody that can focus effector T-cell activity. Proc Natl Acad Sci USA 83, 1453-7.

Staerz, U.D. and Bevan, M.J. (1986) Use of anti-receptor antibodies to focus T cell activity. Immunol Today 7, 241.

Staerz, U.D. and Bevan, M.J. (1989). Redirecting the cellular immune response. Int Rev Immunol 4, 159-73.

Straubinger, R.M., Lopez, N.G., Debs, R.J., Hong, K. and Papahadjopoulos, D. (1988) Liposome-based therapy of human ovarian cancer: parameters determining potency of negatively charged and antibody-targeted liposomes. Cancer Res 48, 5237-45.

Suresh, M.R. (1991). Immunoassays for cancer-associated carbohydrate antigens. Semin Cancer Biol 2, 367-77.

Suresh, M.R., Cuello, A.C. and Milstein, C. (1986a). Advantages of bispecific hybridomas in one-step immunocytochemistry and immunoassays. Proc Natl Acad Sci USA 83, 7989-93.

Suresh, M.R., Cuello, A.C. and Milstein, C. (1986b). Bispecific monoclonal antibodies from hybrid hybridomas. Methods Enzymol 121, 210-28.

Tada, H., Toyoda, Y. and Iwasa, S. (1989). Bispecific antibody-producing hybrid hybridoma and its use in one-step immunoassays for human lymphotoxin. Hybridoma 8, 73-83.

Takahashi, M. and Fuller, S.A. (1988). Production of murine hybrid-hybridomas secreting bispecific monoclonal antibodies for use in urease-based immunoassays. Clin Chem 34, 1693-6.

Tedder, R.S., Yao, J.L. and Anderson, M.J. (1982) The production of monoclonal antibodies to rubella haemagglutinin and their use in antibody capture assays for Rubella specific IgM. J Hyg 88, 335-50.

Thirion, S., Motmans, K., Heyligen, H., Janssens, J., Raus, J. and Vandevyver, C. (1996). Mono- and bispecific single-chain antibody fragments for cancer therapy. Eur J Cancer Prev 5, 507-11.

Tiebout, R.F., Sauerwein, R.W., van der Meer, W.G., van Boxtel-Oosterhof, F. and Zeijlemaker, W.P. (1987). Requirements for growth of Epstein-Barr virus-transformed cells at low cell densities. Immunology 60, 187-93.

Tilcock, C., Ahkong, Q. F. and Fisher, D. (1993) Polymer-derivatized technetium ^{99m}Tc-labeled liposomal blood pool agents for nuclear medicine applications. Biochim Biophys Acta 1148, 77-84.

Truneh, A., Machy, P. and Horan, P.K. (1987) Antibody-bearing liposomes as multicolor immunofluorescent markers for flow cytometry and imaging. J Immunol Methods 100, 59-71.

Tutt, A., Greenman, J., Stevenson, G.T. and Glennie, M.J. (1991a). Bispecific F(ab'gamma)3 antibody derivatives for redirecting unprimed cytotoxic T cells. Eur J Immunol 21, 1351-8.

Tutt, A., Stevenson, G.T. and Glennie, M.J. (1991b). Trispecific F(ab')3 derivatives that use cooperative signaling via the TCR/CD3 complex and CD2 to activate and redirect resting cytotoxic T cells. J Immunol 147, 60-9.

Tyle, P. and Ram, B.P. (1990) Monoclonal antibodies, immunoconjugates, and liposomes as targeted therapeutic system. Targeted Diagn Ther 3, 3-22.

Urnovitz, H.B., Chang, Y., Scott, M., Fleischman, J. and Lynch, R.G. (1988). IgA:IgM and IgA:IgA hybrid hybridomas secrete heteropolymeric immunoglobulins that are polyvalent and bispecific. J Immunol 140, 558-63.

Vaage, J. Barbera-Guillem, E., Abra, R., Huang, A and Working, P. (1994) Tissue distribution and therapeutic effect of intravenous free or encapsulated liposomal doxorubicin on human prostate carcinoma xenografts. Cancer 73, 1478-84.

Valone, F.H., Kaufman, P.A., Guyre, P.M., Lewis, L.D., Memoli, V., Ernstoff, M.S., Wells, W., Barth, R., Deo, Y. and Fisher, J. (1995). Clinical trials of bispecific antibody MDX-210 in women with advanced breast or ovarian cancer that overexpresses HER-2/neu. J Hematother, 4, 471-5.

Van Dijk, J., Tsuruo, T., Segal, D.M., Bolhuis, R.L., Colognola, R., van de Griend, R.J., Fleuren, G.J. and Warnaar, S.O. (1989). Bispecific antibodies reactive with the multidrug-resistance-related glycoprotein and CD3 induce lysis of multidrug-resistant tumor cells. Int J Cancer 44, 738-43.

Van Dijk, J., Zegveld, S.T., Fleuren, G.J. and Warnaar, S.O. (1991). Localization of monoclonal antibody G250 and bispecific monoclonal antibody CD3/G250 in human renal-cell carcinoma xenografts: relative effects of size and affinity. Int J Cancer 48, 738-43.

Van Osdol W.V., Sung C., Dedrick R.L. and Weinstein J.N. (1993) A distributed pharmacokinetic model of two-step imaging and treatment protocols: Application to streptavidin-conjugated monoclonal antibodies and radiolabeled biotin. J Nucl Med 34, 1552-64.

Varma, V.A., Cerjan, C.M., Abbott, K.L. and Hunter, S.B. (1994) Non-isotopic in situ hybridization method for mitochondria in oncocytes. J Histochem Cytochem 42, 273-6.

Vemuri, S. and Rhodes, C.T. (1995) Preparation and characterization of liposomes as therapeutic delivery systems: a review. Pharm Acta Helvetiae 70, 95-111.

Vincent, P. and Samuel, D. (1993) A comparison of the binding of biotin and biotinylated macromolecular ligands to an anti-biotin monoclonal antibody and to streptavidin. J Immunol Methods 165, 177-82.

Vingerhoeds, M.H., Storm, G. and Crommelin, D.J. (1994) Immunoliposomes *in vivo*. Immunomethods 4, 259-72.

Weiner, L.M., Clark, J.I., Ring, D.B. and Alpaugh, R.K. (1995). Clinical development of 2B1, a bispecific murine monoclonal antibody targeting c-erbB-2 and Fc gamma RIII. J Hematother 4, 453-6.

Wilchek, M. and Bayer, E.A. (1984) The avidin-biotin complex in immunology. Immunol Today, 5, 39-43.

Wilchek, M. and Bayer, E.A. (1988) The avidin-biotin complex in bioanalytical applications. Anal Biochem 171, 1-32.

Williams, S.S., Alosco, T.R., Mayhew, E., Lasic, D.D., Martin, F.J. and Bankert, R.B. (1993) Arrest of human lung tumor xenograft growth in severe combined immunodeficient mice using doxorubicin encapsulated in sterically stabilized liposomes. Cancer Res 53, 3964-7.

Winter, G. and Milstein, C. (1991). Man-made antibodies. Nature 349, 293-9.

Wistow, B. W., Subramanian, G. and Van Heertum, R. L. (1977) An evaluation of ^{99m}Tc-labeled hepatobiliary agents. J Nucl Med 18, 455-61.

Wognum, A.W., Lansdorp, P.M., Eaves, A.C. and Krystal, G. (1989). An enzyme-linked immunosorbent assay for erythropoietin using monoclonal antibodies, tetrameric immune complexes, and substrate amplification. Blood 74, 622-8.

Woll, P.J. and Hart, I.R. (1995) Gene therapy for lung cancer. Ann Oncol 6, suppl 1, 73-7.

Wong, J.T. and Colvin, R.B. (1987) Bispecific monoclonal antibodies: selective binding and complement fixation to cells that express two different surface antigens. J Immunol 139, 1369-74.

Woodle, M. C. (1993) Gallium-67-labeled liposomes with prolonged circulation: preparation and potential as nuclear imaging agents. Nucl Med Biol 20, 149-55.

Woodle, M. C. and Lasic D. (1992) D Sterically stabilized liposomes. Biochim Biophys Acta 1113, 171-99.

Wu, M.S., Robbins, J.C., Ponpipom, M.M. and Shen, T.Y. (1981) Modified *in vivo* behavior of liposomes containing synthetic glycolipids. Biochim Biophys Acta 674, 19-29.

Xu, D., Leveugle, B., Kreutz, F.T. and Suresh, M.R. (1998) Mimetic ligand-based affinity purification of immune complexes and immunoconjugates. J Chromatogr B Biomed Sci Appl 706, 217-29.

Yankofsky, S.A., Gurevitch, R., Niv, A., Cohen, G. and Goldstein, L. (1982) Solid phase assay for d-biotin on avidin-cellulosse disks. Anal Biochem 118, 307-14.

Zimmermann, U. and Vienken, J. (1982) Electric field-induced cell-to-cell fusion. J. Membr. Biol. 67, 165-75.